SWEAT GLANDS IN THE SKIN OF THE QUOKKA OF WESTERN AUSTRALIA

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(*Accepted for publication 14th October, 1961.*)

SUMMARY.

It has been found that *Setonyx* possesses apocrine sweat glands in the hairy skin and eccrine glands in the non-hairy parts (paws). It is suggested that maintenance of body temperature is effected through these glands in addition to the usual mechanisms employed by other homoiothermic animals. Licking of the fur does not appear to be a cooling mechanism of any importance.

INTRODUCTION.

In his article on the water metabolism of an Australian marsupial, the quokka (*Setonyx brachyurus*) of Western Australia, Bentley (1955) remarked that "Histological examination of the skin from various parts of the body showed that sweat glands were present only on the fore and hind paws".

In a recent survey of the skin glands of certain other Australian marsupials including the phalanger (*Trichosurus vulpecula*), the dormouse opossum (*Dromicia concinna*), the bandicoot (*Thylacis obesulus*) and the pouched mouse (*Sminthopsis crassicaudata*), Green (in preparation) also found that there are typical eccrine sweat glands in the skin of the fore and hind paws. These glands are, in all four species, well developed and similar to, though somewhat larger than, those found in the palms and soles of man. Serial sections of skin from the crown of the head, interscapular, sternal and inguinal regions, as well as from the ventral and dorsal surfaces of the tail, showed well-developed apocrine sweat glands associated with the hairs. These glands varied in size with their locality. No eccrine sweat glands were present.

Microscopic examination of similar areas of skin from the kangaroo (*Macropus major*) gave similar results.

RESULTS.

In March of this year Mr. Tyndall-Biscoe, of the Physiology Department of the University of Western Australia, very kindly supplied some skin from an adult female quokka (*Setonyx brachyurus*). Areas of skin, similar to those used for the investigation of the other five marsupials, were microscopically examined

LEGENDS TO FIGURES

Fig. 1 (x 120):

A. Surface of skin (crown of head).
B. Hair follicle.
C. Sebaceous gland.
D. Coils of apocrine sweat gland.
E. Duct.

Fig. 2 (x 475):

A. Lumen of coil of sweat gland (base of tail – ventral surface).
B. Secretory cells (in non-secretory phase) of apocrine coil.
C. Duct of apocrine sweat gland.
D. Abrupt change from secretory tubule to duct of apocrine gland.
E. Basement membrane.

Fig. 3 (x 120):

A. Hair in hair follicle (base of tail – dorsal surface).
B. Coil of apocrine sweat gland.
C. Duct of apocrine sweat gland.
D. Opening of duct on to skin surface.

Fig. 4 (x 500):

A. Transverse section of tubule of an apocrine sweat gland (inguinal region).
B. Nuclei of secretory cells.
C. Myoepithelial cell.
D. Longitudinal section of the apocrine tubule.
E. Nuclei of secretory cells.
F. Myoepithelial cell in longitudinal section.
G. Nucleus of myoepithelial cell.

Fig. 1

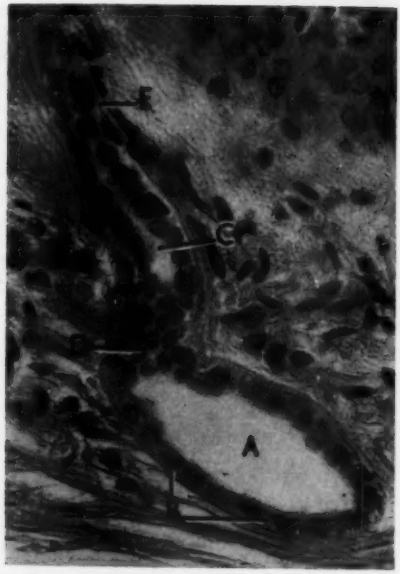

Fig. 2

Fig. 3

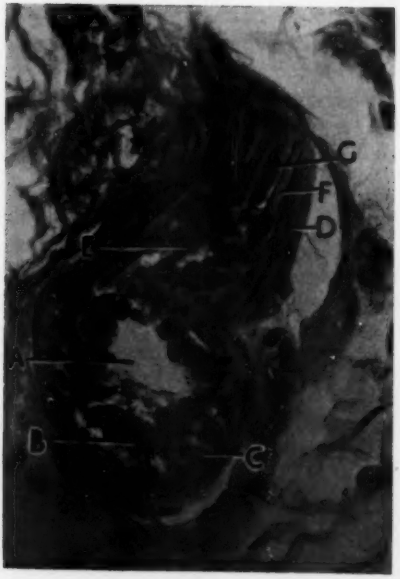

Fig. 4

and as a result it was concluded that there are sweat glands present over the entire body surface of *Setonyx*; apocrine sweat glands associated with hairs and eccrine sweat glands (also see Bentley, 1955) in the non-hairy thickly keratinized skin of the fore and hind paws.

As Bentley (1955) did not mention the presence of apocrine glands in the hairy skin areas of *Setonyx* it is uncertain whether or not he places the apocrines in the category of sudoriferous glands.

Description of the apocrine glands of Setonyx.

These are structures of which the coiled portion tends to appear in the deepest part of the dermis or in the upper portion of the hypodermis. The glands consist of spirally coiled tubules: this description accords with that given by Montagna (1956) who notes that the "Axillary apocrine sweat glands, in man, are usually described as compound tubular or alveolar glands, although wax model reconstructions and teased whole glands from fresh axillary skin show them to be simple, coiled tubular glands".

In *Setonyx*, as well as in the other marsupials that have been examined, the secretory coils are situated at the bases of large hair follicles.

Examination of the various skin areas already mentioned discloses that the secretory part of the tubules makes up to three or four loose spiral turns in the lower dermal region. A capillary investment accompanies the coils. Near the base of a hair follicle, each tubule becomes straight and then narrows to meet the duct (Fig. 1) which can in some cases be seen to open into the pilosebaceous canal of its associated hair (Fig. 1). Other ducts are observed to open into the pilosebaceous canal of the associated hair at the exit of the canal on to the surface of the skin (Fig. 3).

The majority of tubules examined appear to be in the non-secretory phase (Fig. 2), the epithelium being cuboidal or flattened. Those tubules which are in a secretory phase contain tall columnar cells with a dark, short, oval, basal nucleus (Fig. 4). The somewhat granular cell cytoplasm tends to fragment on its luminal border.

Intact cells appear to be divided into three equal sections; a basal third which contains the darkly staining nucleus, a middle third in which the cytoplasm displays a granular appearance and an apical third consisting of pale colourless cytoplasm which forms a rounded protrusion into the lumen (Fig. 4).

Myoepithelial cells form an acidophilic investment around the tubule, their nuclei being placed perpendicular to those of the tubule cells. When viewed in transverse section, in the contracted state, the myoepithelial cells present a closely folded appearance with the nucleus apparently pushed towards the apex of the cell, so bringing it immediately beneath the secretory cells of the tubule. When seen in longitudinal section the myoepithelial cells tend to be fusiform and to contain an elongated centrally placed nucleus (Fig. 4).

A well-marked basement membrane encloses the two types of cells; this membrane is usually particularly clear in those tubules which exhibit a secretory phase.

Many sections give evidence of the abrupt manner in which a secretory tubule becomes narrowed to join its excretory duct (Figs. 2 and 3). Montagna (1956) also mentions this characteristic in human apocrine sweat glands.

The duct wall consists of two layers of cells; the inner ones are somewhat flattened and shaped to conform to the curvature of the tube while the outer cells are more cuboidal in form. The nuclei of the inner cells are slightly flattened but those of the outer are round and centrally placed.

DISCUSSION.

According to Rothman (1954) apocrine glands are phylogenetically older than the eccrine type. In man the latter are distributed over the whole body while the apocrine system has become rudimentary and occurs only in specialized sites.

In their recent paper entitled "The Sweat Glands", Weiner and Hellman (1960) note that "Both the eccrine and apocrine glands subserve a variety of functions and in both the sweating has come to provide for evaporative heat loss, notably in man and the horse. The appearance of body eccrine glands in great density in man is foreshadowed in the great apes. The acquisition of these glands is associated with a great reduction in apocrine glands".

In a communication in which evaporative water loss and temperature regulation in the marsupial *Setonyx brachyurus* are considered Bentley (1960) remarks that "Temperature regulation is of interest with respect to both phylogeny and ecology. Phylogenetically marsupials are an intermediate between the monotremes which are heterothermic and placentals which are homoiotherms (see Davson, 1959)".

It was formerly believed (Martin, 1902) that marsupials were not as efficient homoiotherms as placental animals, but Bentley (1955) has shown that *Setonyx* is quite an efficient homoiotherm. Bartholomew (1956) and Robinson and Morrison (1957) are in agreement with this finding but claim that maintenance of a constant body temperature by marsupials in a high environmental temperature is due to evaporation, after licking, of saliva from their fur.

As a result of an experiment on several animals (*Setonyx*) Bentley concluded that fur-licking is not a factor in maintaining a constant body temperature at high environmental temperature and high relative humidity.

The fact that *Setonyx* does possess apocrine glands in association with the hairs over its entire body surface, as well as the eccrine glands on its fore and hind paws, suggests that all these glands act as the animal's thermo-regulatory apparatus.

Fur-licking by marsupials at high environmental temperatures may be a mechanism to conserve salt which would remain on the fur after the evaporation of the sweat from the apocrine glands. This "salt-licking" would probably help to appease the thirst engendered by evaporative water loss as well as to reduce the actual loss of water.

Fur-licking may also have a mouth cooling effect due to evaporation of the saliva. Another means of local cooling may be effected by the flattening down of the fur by licking and thus reducing the quantity of air trapped between the hairs. This air in cold weather forms a blanket around the body when the hairs are separated to hold it.

Acknowledgments. I am grateful to Professor A. A. Abbie for his helpful suggestions and critical reading of the manuscript.

Miss P. Pilton of the Department of Zoology (University of Adelaide) obtained from Mr. Tyndall-Biscoe of the Department of Physiology (University of Western Australia) the *Setonyx* material. To both these workers I am much indebted. I wish to thank Miss Margaret Smith and Mrs. G. Kempster who were responsible for the microscopic sections and the photographic illustrations.

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THE EVIDENCE FOR THE SURVIVAL AND REGENERATION OF DONOR EPITHELIAL ELEMENTS IN ORTHOTOPICALLY-GRAFTED SKIN HOMO-TRANSPLANTS

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(*Accepted for publication 11th October, 1961.*)

SUMMARY.

The present studies have shown that some epithelial elements of donor origin survive the homograft response and that, provided adequate physical protection, particularly against desiccation, is given to the graft, these elements may regenerate.

Regeneration of follicular epithelium was invariably observed as early as 8 to 10 days after homotransplantation of skin in specifically presensitized mice. Regeneration of both follicular and surface epithelial elements is usually completed by about the 16th day after grafting in these animals. By comparison, epithelial regeneration was delayed, and was observed much less frequently in homografts transferred to non-immune recipients.

Inhibition of the regenerative capacity of the donor tissues by X-irradiation before transplantation prevented or grossly delayed regeneration of both follicular and surface epithelia in the graft area.

An immunological test based upon Mitchison's (1956) design was used to demonstrate the presence of antigens of both donor and host provenance in the regenerated tissues. The presence of donor elements was confirmed by showing that the transfer of appropriately presensitized and normal spleen cells of the recipient strain to X-irradiated mice bearing the regenerated homografts resulted in destruction of the regenerated tissues.

Homograft regeneration was promoted by passive administration of serum obtained from mice hyper-immunized with donor-type spleen cells. This finding suggested that an "enhancement" phenomenon might play an important rôle in the fate of the regenerating tissues.

INTRODUCTION.

In a preliminary communication (Dineen and Szenberg, 1961) evidence was presented which indicated that some elements of donor origin can regenerate following the acute phase of the second-set homograft response, provided that the orthotopically-grafted donor skin tissues are given adequate physical protection during the regenerative phase. This conclusion was based on the histological appearance of the tissues at the site of the protected homograft.

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Most workers in the homograft field have held that when such regeneration occurs it is wholly a function of host epithelium, and support for our own somewhat heterodox view was derived mainly from the following observations: (1) Antigens of donor origin were detected in the regenerated tissues at 16 days when the acute phase of the second-set reaction had already subsided. (2) Inhibition of the regenerative capacity of the donor tissues by X-irradiation before transplantation to homologous recipients prevented regeneration of the grafts. Under these conditions the surgical wound was repaired by the formation of scar tissue, and (3) a pattern of homograft regeneration was observed in recipients passively immunized with specific hyperimmune-serum, which was similar to the events observed, both macroscopically and microscopically following classical second-set reactions, suggesting that a mechanism similar to that postulated for tumour-graft enhancement by Kaliss (1956) might play an important rôle in regeneration of the homografts.

Subsequent work to be reported in the present paper has added more evidence (from both morphological and functional studies) to support our general contention. In addition evidence will be presented which in our opinion shows that epithelial cells compose part (or all) of the regenerating donor cells.

Materials and Methods.

Animals. The mice used in the present study were of the C3H and C57B1 inbred strains obtained by random matings of the progeny of stock lines maintained by brother-sister matings. The homogeneity of genotype within the strains was recently confirmed by the apparent (macroscropic) complete acceptance of both a first and a second orthotopically transplanted skin isograft among 12 randomly selected males of the current three generations of each strain. The mice used in the following studies belonged to these generations. A small group of mice of the inbred AKR strain were also used in the present studies.[2]

Preparation of spleen-cell suspensions. Single-cell suspensions of spleen cells were obtained by finely dicing pooled spleens collected with aseptic precautions into Earle's saline. The diced fragments of spleen were then transferred to a stainless steel gauze sieve and single cells and small clumps of cells were gently expressed from the fragments with a pestle and washed into a sterile Petri dish with an excess volume of Earle's saline. After transfer of the cell suspensions to centrifuge tubes, the small clumps were separated from the single-cell suspensions on standing for 10 minutes at room temperature. The clumps of cells were further dispersed by gentle pipetting for 10 minutes at room temperature in 0·25 p.c. trypsin (Difco) in Earle's saline buffered at pH 7·3 with bicarbonate. The combined single-cell suspensions were centrifuged at 1000 r.p.m. for 7 minutes and the supernatant fluids discarded. The sedimented cells were then resuspended in Earle's saline and the concentration adjusted to 80 x 10^6 nucleated spleen cells per millilitre.

Technique of skin grafting. The method of orthotopically grafting mice with full-thickness skin has been described by Billingham and Medawar (1951) and Brent (1959). This technique was used in the current study and requires no further elaboration. The only modifications made in their method were (1) usually, as indicated in the following text,

[2] These animals were kindly supplied by Dr. Donald Metcalf, Carden Fellow, Anti-Cancer Council of Victoria.

removal of the pressure bandages was delayed until the 16th day or later following transplantation of the skin tissues, and (2) in one experiment relatively small pieces of donor skin (5 millimetres square) were centrally located in large graft beds (15 millimetres square).

Fixation and staining of skin. Specimens of grafted skin together with the underlying panniculus carnosus of the graft beds were extended on a piece of filter paper and fixed in Zenker-formol saline. Sections were routinely stained with Mayer's alum haematoxylin and eosin and with Unna-Pappenheim stain.

X-irradiation of donor skin and whole-body irradiation of recipient mice. The radiation source was a 250 KV Phillips constant potential unit. Whole-body irradiation of the mice was carried out at 250 KV, 15 ma with a dosage rate of 306 r per minute. Total dosage was 800 r and the animals were placed 30 cm. from the target.

Skin grafts removed from donors in the inactive phase of the hair growth cycle were subjected to a total dosage of 1000 r before transplantation to recipient mice. The dosage rate was 156 r per minute and the tissues were irradiated at a distance of 50 cm. from the target.

Immunization of C3H mice for serum "enhancement" factor. A group of adult male C3H mice were injected intraperitoneally with a single-cell suspension of C57B1 spleen containing 20 x 10^6 nucleated cells on three occasions at weekly intervals. The first course of injections was followed one month after the 3rd injection with a second course of injections with C57B1 spleen cells, using the same route and dosage. The animals were exsanguinated by cardiac puncture four days after the 6th injection and the serum separated from the pooled bloods.

The immunological test for the detection of elements of donor provenance in the regenerating tissues. The presence of elements of both donor and host origin in the regenerating tissues of the homografts may be detected by an immunological test based upon the antigenic disparity between host and donor strain. The test is similar in principle to that designed by Mitchison (1956) and modified by Billingham and Brent (1959) to demonstrate chimaeric cells in the lymphoid organs of recipient mice following the homologous transfer of spleen, lymph node and bone marrow cells. In the present study, the need was to demonstrate the presence of elements isogenic either with the recipient strain (usually C3H) or with the donor strain (usually C57B1) in the regenerating tissues. The regenerated grafts were transferred at 16 days to normal adult male C3H and C57B1 mice. These indicator animals were then reciprocally challenged 6-7 days later with C57B1 and C3H homografts. If the challenge graft was rejected with a typical second-set accelerated reaction this was taken as evidence that the regenerating graft which has been implanted on the animal contained cells antigenically similar to those of the challenge graft.

RESULTS.

A Histological Study of the Events Associated with Regeneration of Elements of Donor Genotype in Skin Homografts.

Regeneration of epithelial elements was invariably observed in homografts implanted in presensitized animals and handled as described under Methods, while regeneration of these elements occurred in only a proportion of grafts (less than 20 p.c.) transferred to normal untreated recipients. The first section of this paper is concerned with the histological changes which follow implantation of skin homografts (a) in the presensitized animals and (b) in untreated recipients of the same strain.

The evolution of the homograft response and regeneration in presensitized mice. Thirty-six C3H adult mice (10-12 weeks old) were each injected intraperitoneally three times during a ten-day interval with 20 x 10^6 C57B1 nucleated spleen cells. Four days after the last injection the animals were grafted orthotopically with normal C57B1 skin in the inactive phase of the hair growth cycle. Homografts, graft bed and a section of the adjacent host tissues were removed from three of the sensitized animals at intervals of two days and fixed for sectioning. The grafts remaining at 16 days were unbandaged and were retained macroscopically intact as previously described (Dineen and Szenberg, 1961).

Histological appearance of the grafts. At two days the surface and follicular epithelia of the grafts were intact but no mitotic figures were seen in the sections. A very moderate polymorph infiltration with a few scattered lymphocytes was observed in the demarcating zone between the graft and the panniculus carnosus of the host. The grafts were not revascularized at this early stage. At four days the surface epithelia of the grafts were reduced to one to two layers of cells and the dermis was oedematous and infiltrated with an occasional polymorph. Small dense foci of polymorphs were present in the demarcating zone between graft and host tissues. By the sixth day after transplantation the surface epithelia of the grafts were completely destroyed and some areas were covered by polymorph debris (see Fig. 1). Most of the follicles were necrotic and the surviving follicular epithelium was surrounded by polymorphs. At this stage the dermis, although oedematous, was well vascularized. The demarcating zone between graft and host was characterized by a dense infiltrate containing lymphocytes, plasma cells and fibroblasts and foci of polymorphs.

The most interesting feature observed in sections of the grafts obtained on the 8th day after transplantation was the presence of large nucleoli staining dark red with pyronin in the cells of the surviving follicular epithelium (see Fig. 2). This finding provided the earliest evidence, confirmed in subsequent sections, that the surviving follicles were capable of regenerating. By the 10th to the 12th day after grafting, the graft area was once again covered with 1-2 layers of epithelial cells and the entry of the surviving epithelium into a phase of active regeneration was indicated by the presence of mitotic figures (see Fig. 3). It is also apparent in Fig. 3 that in some areas the follicular and surface epithelia of the grafts are continuous. The demarcating infiltrate, though more intense, was of the same general character as that observed in the tissues sectioned at eight days. Regeneration of the surface epithelium generally appeared to be complete (2-4 layers of cells) in sections of transplants removed 14-16 days after grafting (see Fig. 4). Follicles were numerous in these sections and by the 18th-20th day hair papillae were visible in some of the follicles (see Fig. 5). It was interesting to note that occasional lymphocytes were present in the regenerated epithelial layers. The significance of this finding is discussed below. The demarcating infiltrate containing fibrocytes, polymorphs, lymphocytes and occasional plasma cells was less intense in the specimen taken at 14-16 days and greatly reduced in the 18-20 days specimens.

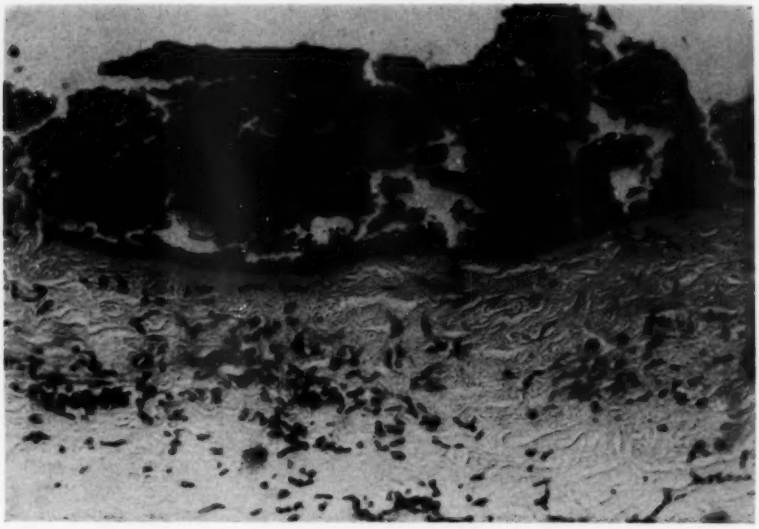


Fig. 1. Second-set homograft reaction – 6 days (x 250).

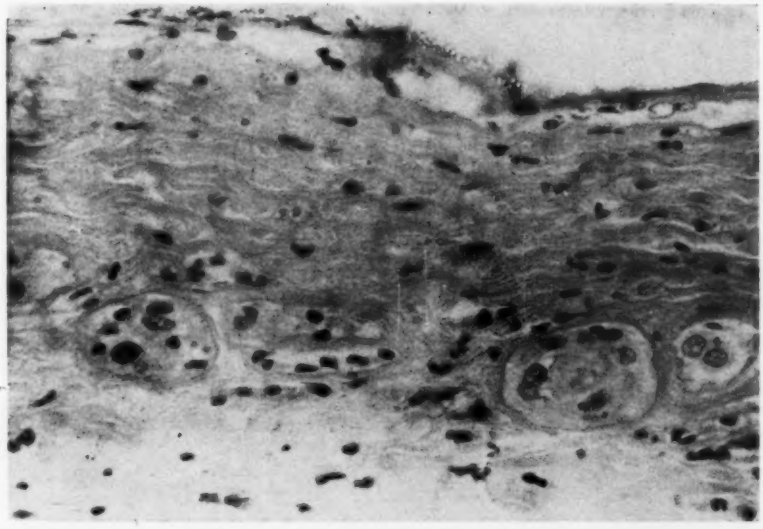


Fig. 2. Early follicular regeneration – 8 days. Note pyronin staining of nucleoli. Unna-Pappenheim stain (x 450).

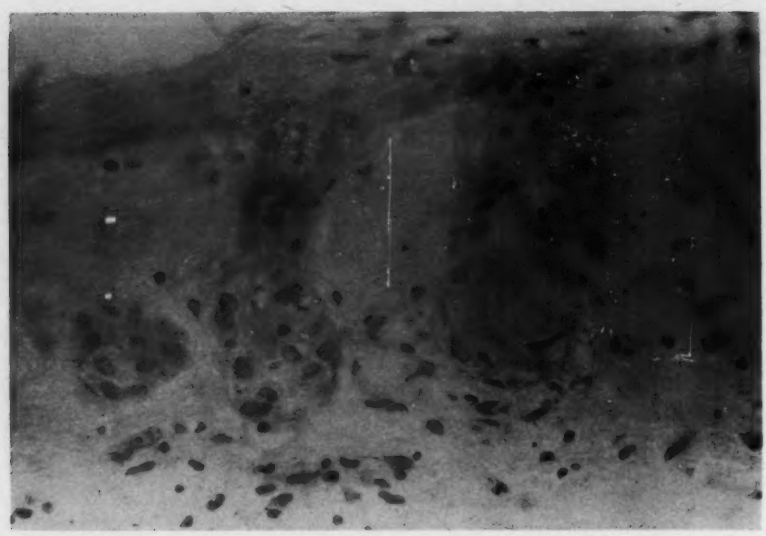


Fig. 3. Regenerating follicles – 12 days. Surface and follicular epithelia continuous. Note mitotic figure in follicular epithelium (x 450).

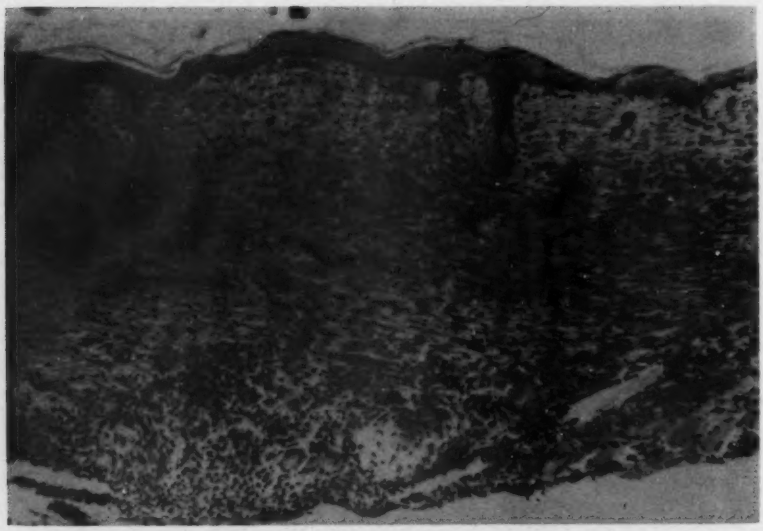


Fig. 4. Regenerated homograft – 16 days (x 100).

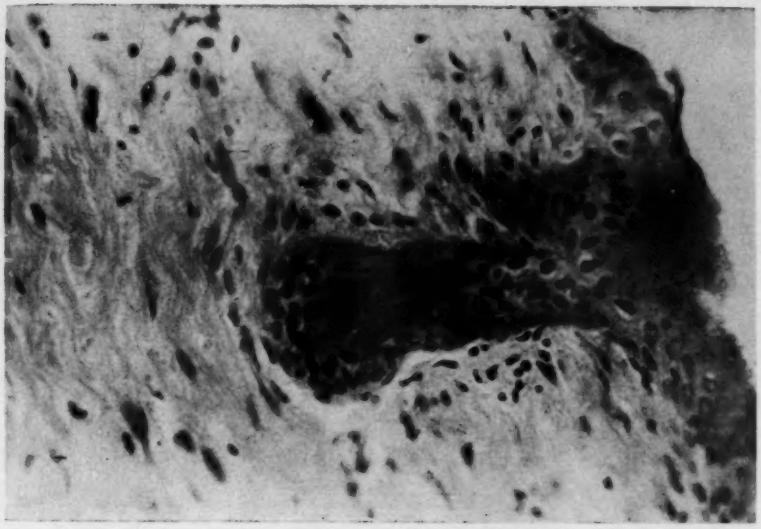


Fig. 5. Regenerating follicle containing developing hair papilla – 20 days (x 450).

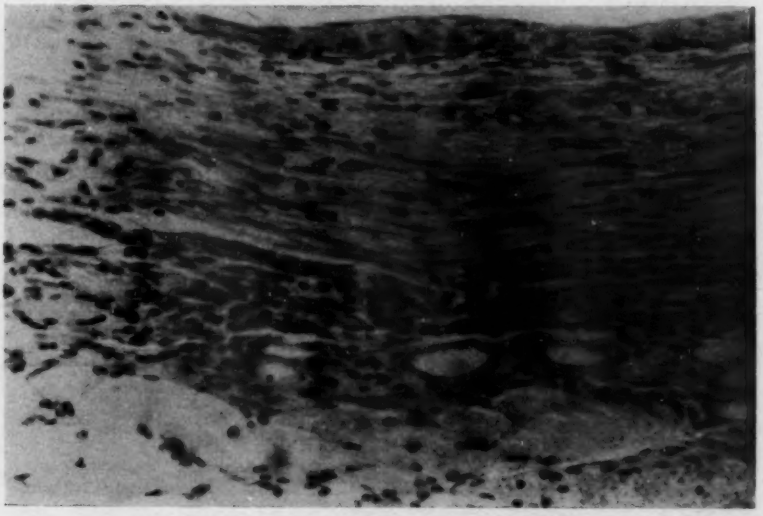


Fig. 6. Scar tissue – 18 days (x 250).

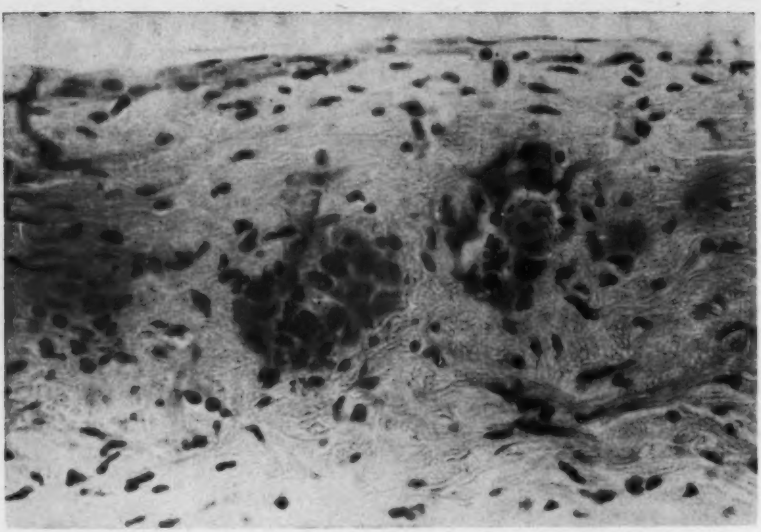


Fig. 7. Early follicular regeneration following first-set homograft reaction — 14 days (x 450).

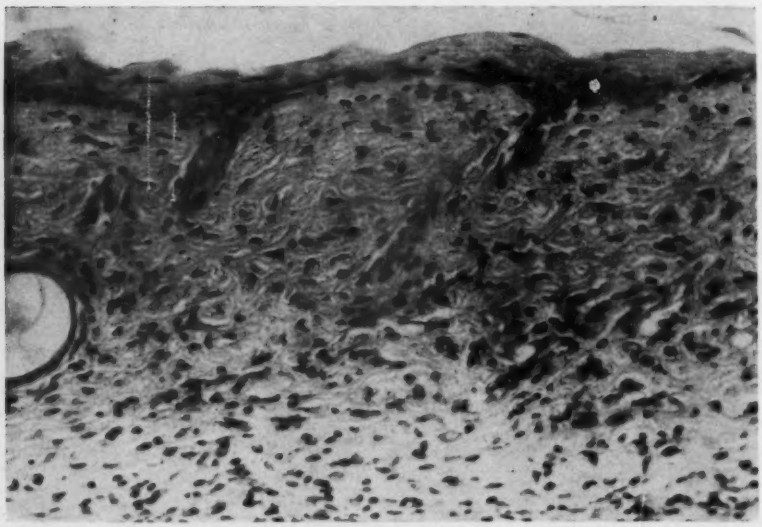
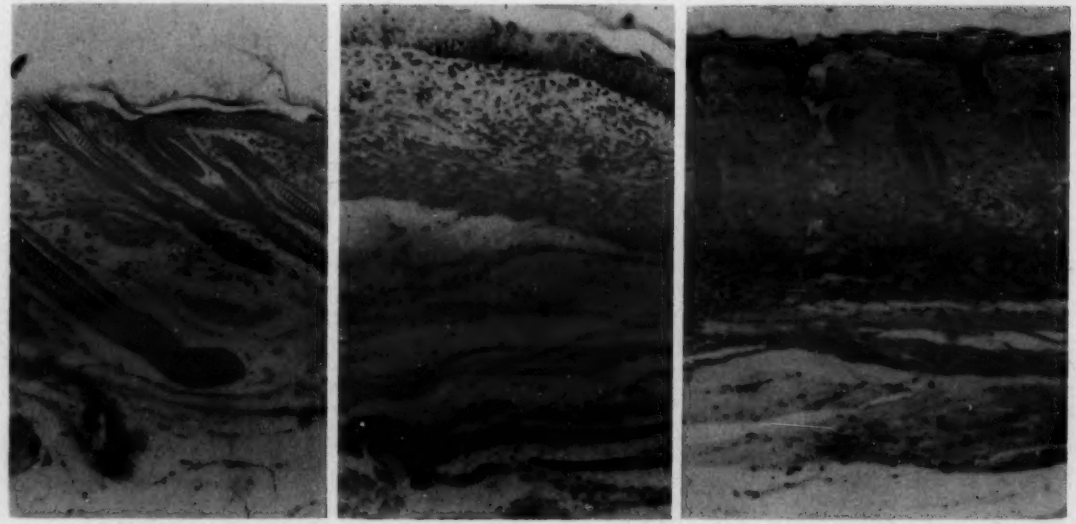


Fig. 8. Regenerated homograft following first-set reaction — 18 days. Note mild lymphocytic response (x 250).



(a) (b) (c)

Fig. 9. Composite Section – 16 days.

(a) Adjacent host tissue.

(b) Cellular scar tissue over-grown by host epithelium.

(c) Graft containing regenerating follicles (x 100).

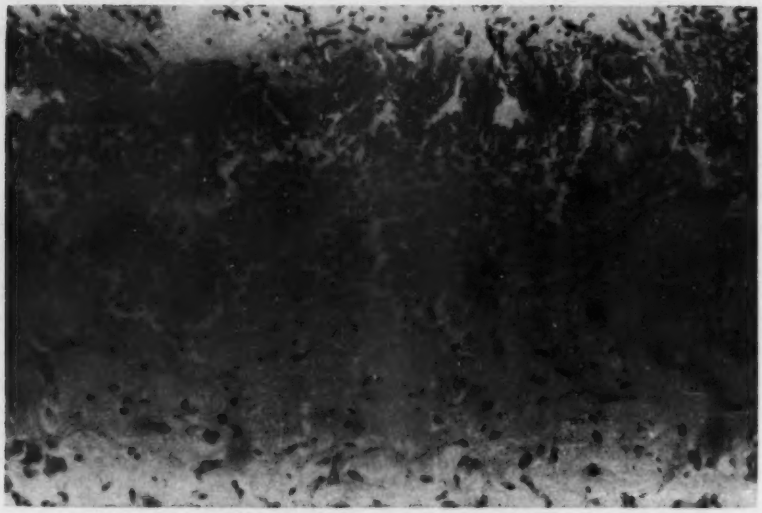


Fig. 10. Gross destruction of X-irradiated homograft – 17 days. Note early regeneration of two follicles (x 250).

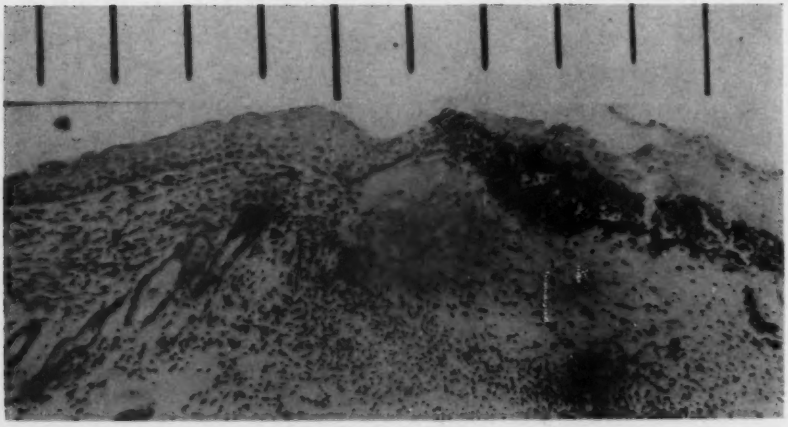


Fig. 11. Invasion of X-irradiated graft by host epithelium – 17 days. 1 div. = 0·1 mm. (x 100).

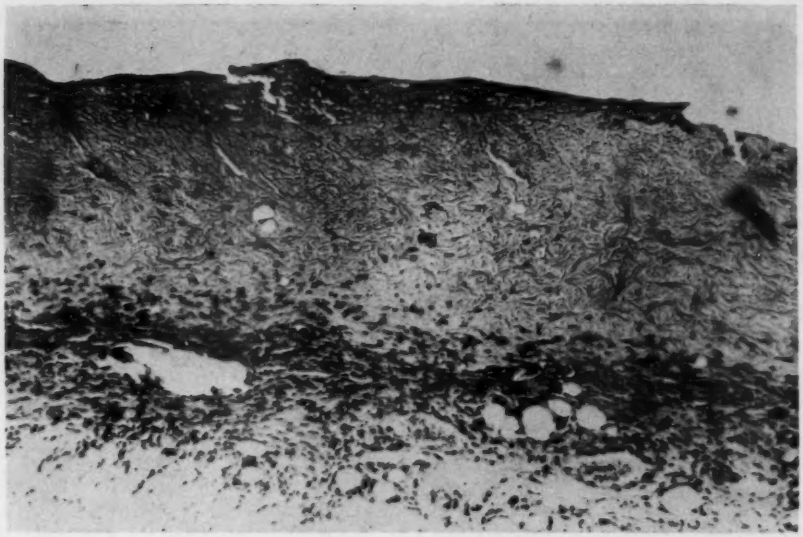


Fig. 12. Destruction of regenerating homograft following the injection of presensitized spleen cells of the recipient (C3H) strain – 4 days after cell transfer (x 100).

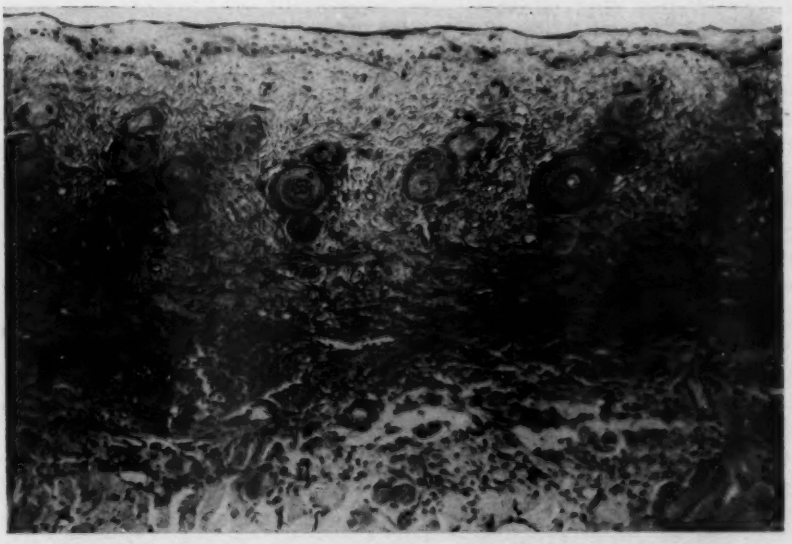


Fig. 13. Regenerating homograft following the injection of normal recipient-strain spleen cells – 4 days after cell transfer (x 100).

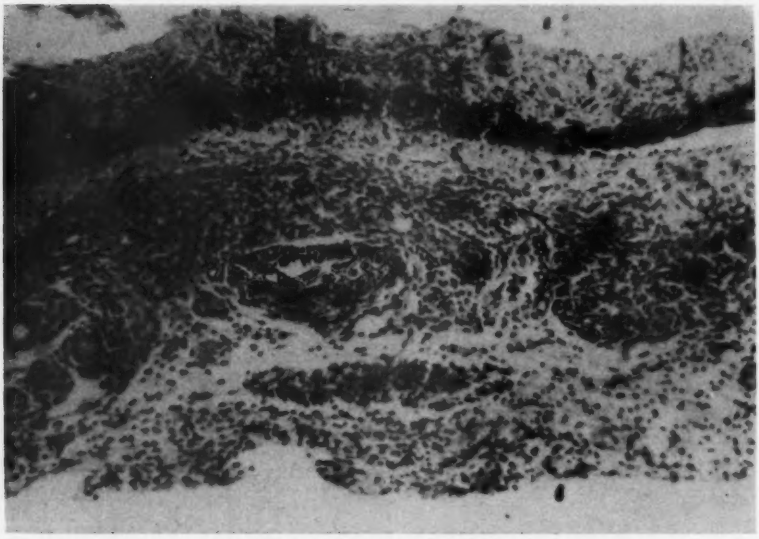


Fig. 14. Destruction of surface and follicular epithelium of homograft in recipient treated with normal serum – 16 days (x 100).

Fig. 15. The effect of "enhancement". Surface and follicular epithelial regeneration of homograft in recipient treated with immune serum–16 days (x 100).

The evolution of the homograft response in normal mice.

Twenty-four C3H adult male mice were grafted with C57B1 skin. Homografts, underlying panniculus carnosus and the adjacent host tissues were removed from three recipient mice at 4-6 days, 8 days, 12-16 days and 18 days after transplantation. Unless the transplants were previously removed for sectioning, the pressure bandages were retained until the 16th day after grafting.

At 4-6 days the follicular epithelia of the grafts were intact and mitotic figures were prominent in the epithelia of the tissues sectioned at six days. By six days the grafts were well vascularized and a few polymorphs were present in the boundary zone between graft tissue and the panniculus of the host. Continued growth of the original transplanted epithelium was observed in the 8th day sections, and lymphocytes and polymorphs were scattered throughout the dermis. Some lymphocytes had penetrated the epithelial layers. The demarcating infiltrate was well developed at this stage and contained fibroblasts, lymphocytes and foci of polymorphs. At 10 days the surface epithelium of the grafts was completely destroyed and only remnants of the follicular epithelium remained intact. The sub-epithelial zone was densely infiltrated with polymorphs, and lymphocytes and polymorphs were scattered throughout the dermis. The demarcating infiltrate was most conspicuous at this stage and contained many plasma cells, lymphocytes, fibroblasts and polymorphs. By the 12th to the 14th day after grafting it was apparent that subsequent events may follow either one of two separate courses. Usually repair of the graft was brought about by invasion of the graft area by epithelial elements derived from the host and the formation of scar tissue. In this event complete overgrowth of the graft area by host epithelium was delayed until about the 18th day after grafting as shown in Fig. 6. In the remaining grafts (less than 20 p.c.) 1-2 layers of epithelium were already present in the 12-day specimens and early regeneration of the epithelium of surviving follicular elements was indicated by the presence of mitotic figures (see Fig. 7). At 18 days the follicles were well developed and the entire graft area was covered by 2-4 layers of epithelial cells. Although the development of follicles and the absence of scar tissue were the main features which characterized this second course of events it was also apparent that a mild lymphocytic infiltration had occurred in the dermis of the regenerating tissues by about the 12th day, and invasion of the epithelial layers was also observed in the 18-day specimens (see Fig. 8). This late lymphocytic response was not observed in scar tissue. The significance of these findings is discussed below.

THE DIFFERENTIATION OF HOST AND DONOR TISSUE RESPONSES — SMALL GRAFTS CENTRALLY LOCATED IN LARGE GRAFT BEDS.

In an earlier study of the homograft response in rabbits, Medawar (1944) used the simple device of placing small skin grafts in relatively large graft beds to investigate the rôle of the ingrowth of host epithelium during repair of the graft area. This design was adopted in the present study in an attempt

to distinguish between the contributions to epithelial repair of the graft area due to the ingrowth of host tissues, and to regeneration of epithelial elements of donor origin. Accordingly, 10 C3H mice were immunized with C57B1 spleen cells as previously described and grafted with C57B1 skin. The grafts which were approximately 5 mm. square were placed in graft beds 15 mm. square. The grafts, surrounding wound area and a section of the normal host skin were fixed for sectioning on the 16th day after grafting. Three distinct areas were recognized on microscopic examination of the 16-day specimens (see Fig. 9). The first area of host tissue contained mature epithelium, follicles containing hair, and normal dermis. The second area, situated between the normal host tissue and the graft area proper, contained 3-4 layers of epithelial cells overlying typical young scar tissue including fibrocytes and capillaries, and collagen fibrilles aligned parallel to the superficial epithelium. The third area contained two to four layers of epithelial cells, regenerating follicles and normal connective tissue. Single polymorphs and lymphocytes were scattered throughout the dermis and epithelium. This graft area was separated from both scar tissue and host panniculus by a dense demarcating infiltration of round cells, fibrocytes and fibroblasts.

Immunological Demonstration of the Presence of Antigens of both Donor and Host Genotype in the Regenerating Tissues.

A summary of the experiments designed to show the presence of elements of both donor and host origin in the regenerated tissues is presented in Table 1. The recipient animals used to provide the regenerating grafts had been presensitized with spleen cells of the skin-donor strain. The regenerated tissues were transferred to indicator mice at 16 to 18 days after grafting as the histological studies showed that the destructive phase of the homograft response had subsided, and that regeneration was either advanced or complete, at this stage in presensitized animals. Indicator mice were challenged with a skin graft of the appropriate strain six or seven days after transfer of the regenerated tissues and the extent of destruction of the challenge grafts was assessed at six days. In two experiments (see Table 1, c and d) the possibility that presensitization of the recipient mice might be "adoptively" acquired (*cf.* Mitchison, 1953, and Billingham, Brent and Medawar, 1954) by the coincidental transfer of isologous, immunologically competent cells present in the regenerated tissues, was excluded by the introduction of a third strain of mice (AKR) as the indicator animals. Under these conditions[3] coincidentally transferred lymphoid cells would not be expected to survive. Microscopic inspection of the challenge grafts was carried out at six days after implantation. The criteria used for definition of a second-set homograft response were—(a) destruction of both follicular and surface

[3] Although it is known that both the C3H and AKR strains are genetically related ($H\text{-}2^k$) orthotopic skin grafts exchanged between individuals of these strains are rejected in the normal way.

TABLE 1.

The antigenic provenance of the regenerating homografts.

Strain of skin donor	*Recipient strain and No.	Time of transfer of regenerating graft and strain of "indicator" recipient	Strain of donor of challenge graft	Classification of the immune response evoked by the challenge graft
a. C57B1	10×C3H	C3H—16 days	C57B1	Second-set homograft response
b. C57B1	6×C3H	C57B1—17 days	C3H	Second-set homograft response
c. C57B1	6×C3H	AKR—16 days	C57B1	Second-set homograft response
d. C57B1	6×C3H	AKR—16 days	C3H	Second-set homograft response
e. C57B1	10×AKR	AKR—18 days	C57B1	Second-set homograft response

* All recipient animals were injected intraperitoneally with 20×10^6 nucleated spleen cells of the skin-donor strain 3 times during the 10-day period grafting.

epithelia, (b) the development of an intense sub-epithelial polymorph infiltrate, and (c) the development of a combined polymorph and round-cell infiltrate in the demarcating zone between the graft and host tissues.

The results summarized in Table 1 provide *prima facie* evidence that elements of both donor (Table 1 a, c and e) and host origin (Table 1 b and d) are present in the regenerated tissues and can sensitize the indicator recipients to give a second-set homograft response.

The Effect of X-irradiation of Donor Skin on Homograft Regeneration.

Both the morphological events which were observed during the regeneration of the homografts, and the detection of cellular antigens of donor type in the regenerated grafts suggested that multiplication of donor elements might play an important rôle in the type of homograft regeneration which was observed during the present studies. If the multiplication of donor components is essential, then regeneration of the graft should be inhibited by X-irradiation of the donor tissues prior to transplantation as this treatment would suppress the mitotic activity of the transplant, though the reparative capacity of the elements derived from the host should remain unimpaired. Accordingly, 24 C3H adult male mice were grafted with X-irradiated C57B1 skin (total dosage 1000 r). The plaster bandages were removed on the 16th day after grafting, and grafts were removed from six of the mice on the 17th day and fixed for sectioning. The remaining grafts dried out and scabbed during the following 48 hours with the formation of scar tissue.

Complete destruction of the surface epithelium and of a large part of the follicular epithelium and the connective tissue was observed in histological sections of the 17-day grafts and regeneration of the follicular epithelium was either absent or grossly delayed (see Fig. 10). A dense sub-epithelial infiltration of polymorphs was seen in the sections and the demarcating zone between graft and host tissues was characterized by an infiltrate containing polymorphs, fibro-

blasts and round cells, including lymphocytes and occasional plasma cells. Invasion of the graft area under the connective tissues by epithelial elements derived from the adjacent tissues of the host was also observed. The extent of ingrowth by host epithelium (about 0·1 mm.) is shown in Fig. 11.

"Adoptive" Activity Against the Regenerating Tissues.

Billingham, Brent and Medawar (1954) have shown that an orthotopic skin homotransplant surviving on an appropriate, immunologically tolerant recipient may be destroyed following the injection of lymphoid cells obtained from normal animals of the recipient strain. The transfer of immunological responsiveness of this type has been designated "adoptive" immunity by these workers, to distinguish this cellular responsiveness from those types of immunity acquired either by passive transfer of serum antibodies, or by direct antigenic stimulation. This design was used in the present studies to show that the regenerating tissues were destroyed by parenteral injection of the host with immunologically competent cells of the same (graft-recipient) strain.

Twenty-four C3H male mice were presensitized with C57B1 spleen cells and grafted with C57B1 skin as previously described. The mice were exposed to lethal, whole-body X-irradiation (800 r) on the 10th day after grafting. Graft areas were shielded with lead strips 2 mm. thick during irradiation. Immediately after irradiation 12 of the grafted animals were each injected intraperitoneally with a cell suspension containing 80 x 10^6 nucleated spleen cells and 30 x 10^6 nucleated bone marrow cells obtained from adult C3H mice immunized with C57B1 spleen cells. The remaining animals were similarly treated with spleen and bone marrow cells obtained from normal adult C3H mice. Grafts and adjacent host tissue were removed from three animals in each of the two groups at 14, 16 and 19 days after grafting, i.e. at 4, 6 and 9 days after injection of the isologously-transferred lymphoid cells.

Microscopic examination of the graft shows that the regenerating tissues were destroyed both in the group of mice injected with presensitized lymphoid cells, and in the group injected with lymphoid cells obtained from normal C3H mice. However, the evolution of the destructive response was accelerated by about two days in the group treated with presensitized cells. Thus the histological picture presented by the six-day (16 days after grafting) sections obtained from the group injected with normal cells was similar to that observed in the group treated with presensitized cells at 4-days after transfer (see Fig. 12). This accelerated responsiveness observed in the animals treated with presensitized cells provides a cogent argument for the contention that destruction of the regenerated tissues was due to an immunological attack by the transferred lymphoid cells. The surface epithelia and most of the follicular epithelia were destroyed in these sections. Lymphocytes and polymorphs were scattered throughout the dermis and the demarcating infiltrate between graft and host

panniculus, which contained fibroblasts, lymphocytes and a few polymorphs, was well developed. Mitotic figures were present in the epithelia of the host tissues and many of the nuclei were pyknotic. By the 6th day after transfer of the presensitized cells, early regeneration of both surface and follicular epithelia was observed in the graft area. A few plasma cells were observed in the demarcating zone in these sections. Regeneration of follicular and surface epithelia was advanced in both groups by the 9th day after cell transfer. Lymphocytes were scattered throughout the dermis and epithelium, and the demarcating infiltrate was predominantly fibrocytic with a few lymphocytes.

One of the most interesting findings of this study was provided by the amount of epithelial regeneration observed in the sections of grafts obtained at four days after transfer of normal C3H lymphoid cells. At this stage the response of the host was presumably destroyed by X-irradiation and the effect of the transferred spleen cells was not yet apparent. The graft area was covered by 4-6 layers of epithelial cells and numerous follicles were present. The actively regenerating epithelial elements contained many mitotic figures. Single polymorphs were observed in both dermis and epithelium, and the demarcating infiltrates contained many fibrocytes and occasional lymphocytes and polymorphs. These observations are illustrated in Fig. 13. The amount of epithelial regeneration observed in these sections was greater than the amount usually seen in homografts obtained from un-irradiated recipients.

Passive Protection of the Regenerated Tissues with Immune Serum.

Twenty C3H adult mice were grafted with C57B1 skin. Each animal was injected intraperitoneally on alternate days from the 6th to the 20th day after grafting with 0·2 ml. of pooled serum obtained from C3H mice hyper-immunised with C57Bi spleen cells (*vide* Materials and Methods—C3H serum "enhancement" factor). A control group (20 C3H adult male mice) was grafted with C57B1 skin and similarly treated with pooled serum obtained from normal C3H mice. The grafts of both the treated and the control groups were inspected on the 16th day after transplantation.

Macroscopically the epithelium of 16/20 grafts in the group treated with hyper-immune serum was completely regenerated, while the epithelium in the control group was completely destroyed in 14/20 grafts and only partially regenerated in the remaining grafts. These observations were confirmed by microscopic examination of sections of the 16-day grafts. Usually complete destruction of both follicular and surface epithelium and most of the graft tissue was observed in the control sections, one example of which is shown in Fig. 14. By contrast, 3-6 layers of epithelial cells and many follicles were present in sections obtained from the mice treated with hyper-immune serum. Occasional lymphocytes were observed in the superficial epithelial layers and foci of polymorphs were present in the dermis. A dense infiltrate containing lymphocytes, plasma cells and connective tissue elements was present in the demarcating zone between

graft and host panniculus. Both features can be seen in Fig. 15. It is apparent, therefore, that administration of serum obtained from specifically presensitized C3H mice exerted a protective ("enhancing") effect upon regeneration of the homografts.

DISCUSSION.

Following Medawar's classical studies of the early and mid-forties, the full thickness orthotopic skin graft has become firmly established as the system of choice for study of the homograft response. Also in line with the procedure devised by Medawar and his colleagues it has become standard practice among students of Transplantation Immunity to remove the pressure bandages protecting the newly grafted tissues at about nine days after transplantation of a first homograft, and at six days on those occasions when an accelerated, second-set homograft response is expected. In the present studies this procedure has been modified by retaining the pressure bandages intact for a much longer period – usually for 16 days following transplantation of the skin graft. The effect of this modification was to provide the grafted tissues with adequate physical protection, particularly against desiccation, during the crucial regenerative period immediately following the acute phase of the homograft response. The following discussion is largely concerned with a critical appraisal of the evidence that some epithelial elements of donor origin survive the acute phase of the homograft reaction, and that these elements not only regenerate but also retain their antigenic character.

The histological evidence that some elements of donor epithelium survive the homograft response and regenerate is based upon the observation that in all grafts transplanted to specifically presensitized animals, pyroninophilia of some epithelial elements at eight days was followed by mitotic activity of these elements. This cellular activity heralds a phase of active epithelial regeneration during the following ten days which frequently, though not inevitably, culminates in the production of hair papillae in the regenerating follicles. The process is most clearly demonstrated when small tissue transplants are centrally located in large graft beds. In sections from such preparations normal host skin containing many mature follicles extending deeply into the dermis, typical young scar tissue overgrown by host epithelium, and graft area containing relatively superficial follicles, form sharply defined zones (see Fig. 9). Although it is apparent that the regenerating follicles are wholly derived from elements of donor origin, it is more difficult to assess the relative rôles played by donor-cell regeneration on the one hand and invasion by host epithelium on the other in development of the superficial epithelial layers which are already prominent in the graft area by the 10th to the 12th day after transplantation.

Our results do not exclude the possibility that most or all of the surface epithelium is derived from cells which survived the initial attack as follicular

epithelium. The fact that in X-irradiated grafts the only evidence of survival was to be seen in some of the follicles seems to indicate that such cells have a high degree of resistance to a variety of insults. It is, however, immaterial where the superficial epithelium arises provided we can establish its donor quality.

In our earlier communication the presence of a significant component of donor origin was demonstrated by showing that the regenerating tissues were rejected by a typical homograft reaction on transfer to normal mice of *both* the donor and recipient strains. An elaboration of this approach has been used in later experiments and the presence of donor elements confirmed by showing that the recipient animals (the indicator mice), having rejected grafts of the regenerating tissues, were able to reject a second challenge graft of the donor strain with a typical second-set reaction (see Table 1).

In a related approach we made use of adoptive immunization to show that the regenerating graft could be attacked by appropriately presensitized lymphoid cells of the host strain. For these experiments, mice bearing the regenerating homografts were heavily X-irradiated (with shielding of the graft area) and then given a large dose of spleen cells from an immunized donor of the host strain. If we accept the dogma that lymphoid cells cannot react immunologically against isogenic tissues the histological findings (see Figs. 12 and 13) must signify that some elements of the graft are of donor (C57B1) origin.

From our results we would conclude that homograft rejection or regeneration is a more complex matter than is usually assumed. It is becoming evident that the induction of perinatal tolerance is not the only manipulation which will permit the persistence of a homograft. For immunological rejection to occur we require (a) movement of foreign antigen from the graft to lymphoid areas, (b) the presence of lymphoid cells capable of recognising the antigen as foreign and responding thereto presumably by proliferation to give a population of immunologically competent cells, (c) access of such cells to the graft, (d) vulnerability of graft cells and tissues to contact with immunologically competent cells and/or cytotoxic antibodies. A block at any of these points can conceivably allow the partial or complete acceptance of the homograft.

We see no reason to assume that adaptation of donor tissues in Woodruff's (1959) sense, i.e. by loss of antigenic character, has played any part in our results. Several of our observations are based directly on the retention of donor antigenicity.

It is well known that with strains that differ only by a minor histo-compatability factor a first homograft may be accepted whereas a second graft from the same donor is frequently rejected without any damage to the first. In this case we have evidence of the diminished vulnerability of antigenically foreign cells which are well established in the host tissues.

We believe that our results are what can be expected from the interaction of the four processes mentioned above and do not require any modification of established immunological principles. In essence we have diminished the

vulnerability of the donor cells by protecting the graft mechanically and concomitantly allowed the build up of a sufficient concentration of "enhancing" antibody to have a significant influence on the flow of "antigenic information" from the graft. Neither is sufficient in itself to allow complete acceptance and we would reiterate that we have never seen *complete* regeneration of the homograft with normally pigmented hair in these experiments. In addition there are several findings which suggest the persistence of a mild chronic immunological response against the regenerated tissue; (a) a mild but persistent lymphocytic infiltration is frequently observed in the epithelial layers of the regenerated tissues, and (b) epithelial regeneration was more active in tissues transplanted in recipients in which the chronic immune response was presumably destroyed by lethal, wholebody X-irradiation administered at 10 days after grafting, than in homo-transplants in un-irradiated, presensitized recipients (see Fig. 13).

Acknowledgments. We are greatly indebted to Sir Macfarlane Burnet, O.M., F.R.S., for his interest and constructive criticism during the course of these studies, and to Miss Jenny Milner and Miss Lois Hawkins for their valuable technical assistance. Our thanks are also due to the staff of the Peter MacCallum Clinic, Melbourne, who X-irradiated the animals and tissues, and to Mr. Andrew Abbot who prepared the photographs.

These studies were supported by grants made available by the National Health and Medical Research Council, Canberra, A.C.T.

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QUANTITATIVE ASPECTS OF THE SIMONSEN PHENOMENON

III. THE EFFECT OF IMMUNIZATION OF THE DONOR FOWL[1]

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(*Accepted for publication 16th October, 1961.*)

SUMMARY.

Immunization of adult fowls with embryonic tissue does not increase the ability of their blood large lymphocytes to produce lesions on the chorio-allantoic membrane. The results of Coombs' tests and of haemagglutination tests indicate that inoculation of spleens from 16-18 day embryos provides an antigenic stimulus in the adult fowl, but that 12-day embryonic spleens evoke no detectable immune response. The bearing of these findings on the application of the clonal selection theory of acquired immunity to the Simonsen phenomenon is discussed.

INTRODUCTION.

The occurrence of splenomegaly in chick embryos following chorio-allantoic transplant of small fragments of adult fowl splenic tissue has been known for many years (Murphy, 1916; Dantchakoff, 1918; Ebert, 1954). In 1957 Simonsen reinvestigated the phenomena. He found that chickens which had been inoculated intravenously with adult fowl spleen cells, or peripheral blood, 3-4 days before hatching usually died in their first or second week after developing a severe anaemia almost invariably associated with a strongly positive Coombs' reaction. Frequently these birds were found to have grossly enlarged spleens. Simonsen concluded that both the splenomegaly and the haemolytic anaemia were due to the proliferation of the adult donor cells in the immunologically immature host and that these phenomena were manifestations of the graft versus host reaction.

Simonsen's findings were extended by Burnet and Boyer (1961) who showed that lesions similar to those observed by Simonsen in the embryonic spleen, following intravenous inoculation of adult fowl lymphoid cells, appeared on the chorio-allantoic membrane following direct inoculation of either white blood cells or spleen suspensions from adult fowls. Burnet and Burnet (1961) found

[1] This work was aided by a grant from the National Health and Medical Research Council, Canberra.

that cells of inbred fowls did not produce any reaction when inoculated into embryos of the same strain but that even a very small genetic difference between host and donor would result in a full response.

It has been established that the cells responsible for the production of lesions on the chorio-allantoic membrane (CAM) are the large and medium lymphocytes (LML) and that an average of 2000 LML is necessary to produce a single lesion (Szenberg and Warner, 1961.) The cell per focus ratio is fairly stable for the same donor and for all donors of same inbred line.

In the present study an attempt was made to establish whether immunization of the donor fowl with embryonic chicken tissue would decrease the cell per focus ratio as compared with that obtained prior to immunization. Some experiments along these lines have already been carried out by Mun, Kosin and Sato (1959) who tested the ability of spleens from adult fowls, pre-inoculated with embryonic splenic tissue, to produce splenomegaly in chick embryos. They could detect no enhancement of the activity of these spleens as compared with spleens from normal fowls but were doubtful as to the immunizing efficiency of their antigens. In view of the finding of Szenberg and Warner (1961) that blood is more efficient than spleen in causing the development of chorio-allantoic foci and since it is now possible to standardize the blood inocula in terms of their LML content it seemed worthwhile to repeat these experiments. An attempt was also made to test the immune status of the donor fowls.

Materials and Methods.

The materials and methods used were essentially those employed by Szenberg and Warner (1962); the strains of outbred fowls and chick embryos used, the method of preparation of blood cells for inoculation of embryos and the technique of obtaining total and differential blood cell and spleen cell counts are fully described in their paper. Chorio-allantoic inoculation of chick embryos was carried out by the method of Burnet and Boyer (1961) and intravenous inoculation by the method of Beveridge and Burnet (1946).

Immunization procedures.

Spleens from 20 or more embryos of the stated age were teased apart with dissecting needles then pressed through fine wire mesh into 2-3 ml. buffered saline (pH 7·4) containing antibiotic (100 units of penicillin and 50 units of streptomycin per ml.). Large clumps were allowed to settle under gravity for 1-2 minutes and the supernatant fluid containing single cells and small cell clumps was used as antigen. Freshly prepared spleen cell suspensions were used for each inoculation.

Adult white leghorn fowls were inoculated intravenously with 0·5 ml. of spleen cell suspension and, at the same time, intraperitoneally with 1 ml. of the suspension emulsified with 1 ml. of Freund adjuvant. The two inoculations were repeated one week later and in some cases repeated again after a further week. The fowls were then left for periods ranging from two to six weeks before testing. Approximately five days prior to testing 0·3 ml. of spleen suspension, prepared in the usual way, was inoculated intravenously. When several successive tests were carried out on the same fowl each test was preceded by an intravenous stimulus approximately five days before bleeding.

Coombs' test.

Serum for use in the direct Coombs' test was prepared by immunizing rabbits with the γ-globulin fraction of fowl serum prepared by methanol precipitation according to the method of Dubert, Slizewicz, Rebeyrotte and Macheboeuf (1953). The inactivated serum was adsorbed overnight at 4° C. with an equal volume of packed, pooled fowl erythrocytes and the absence of heterospecific fowl cell agglutinins was confirmed before use. Normal serum from the same rabbits was treated in the same way.

Chickens were bled from the jugular vein into citrate saline. The cells were washed four times with saline warmed to 37° C. and saline was then added to give a final cell concentration of 35-40 p.c. Slide agglutination tests were carried out by adding one drop of cell suspension to one drop of saline and to one drop of each of two suitable dilutions of Coombs' serum (usually 1/10 and 1/50). The occurrence of agglutination was determined after 10 minutes at room temperature by transilluminating the slide and examining the cells through a low power lens. Normal rabbit serum similarly absorbed with fowl erythrocytes was included among the reagents from time to time as an additional control but it was never found to cause agglutination.

Reactions were graded negative, weak, moderate and strong.

Haemagglutination tests.

Red blood cells from adult fowls were collected in citrate saline either by bleeding from the wing vein or by cardiac puncture. Embryonic red blood cells were collected by opening the egg at the air space end, lifting off the underlying portion of the chorio-allantoic membrane and cutting the allantoic vein. After a few minutes the allantoic fluid containing the extravasated blood was harvested and the cells removed by centrifugation.

The cells were washed three times with saline and made up to a 1 p.c. suspension in saline.

Doubling dilutions of serum were prepared in saline and to each dilution an equal volume (0·25 ml.) of 1 p.c. cell suspension was added. The results were read after the cells had settled for 45 minutes at room temperature. The haemagglutination titre of the serum was expressed as the reciprocal of the dilution causing a standard degree of partial agglutination under these conditions.

EXPERIMENTAL.

Influence of immunization of donor fowls on the CAM focal count.

Immunization of an outbred fowl with tissue suspensions from inbred (AA) embryos.

A suspension of white blood cells from an adult outbred fowl (Bleed I) was titrated for its ability to produce foci on the CAM of both random embryos and embryos of the AA inbred line (Burnet and Burnet, 1961). The fowl was then inoculated intravenously and intraperitoneally with a suspension of pooled spleen, thymus and liver tissue from 15-day AA embryos and the two inoculations were repeated one week later. The fowl was bled for titration seven days after the last dose (Bleed II).

Two weeks after Bleed II a second course of two sets of inoculations of AA tissue suspension at an interval of a week was given and the fowl again bled one week after the last inoculation (Bleed III). Twelve days after Bleed III a

further small inoculum was given intravenously. The fowl was bled again six days later and its white blood cells again titrated on both inbred and outbred embryos (Bleed IV). The results of these titrations are shown in Table 1. It should be noted that in this experiment a suspension of white blood cells was used for titration instead of whole blood. This was obtained by suspending the buffy coat, obtained after centrifugation of the heparinized blood, in buffered saline. The cell content of the inocula is expressed in terms of total white cell count. Differential cell counts were not made.

TABLE 1.

Effect of immunization of a random donor fowl with inbred (AA) tissue on the ability of its white blood cells to produce CAM foci on outbred and AA embryos.

Bleed No.	Total white cells inoculated per embryo	Median focal count	
		AA embryos	Outbred embryos
I (preimmunisation)	$0{\cdot}3\times10^5$	0	1
	$1{\cdot}7\times10^5$	0	4
	$8{\cdot}4\times10^5$	5·0	20
II	$0{\cdot}3\times10^5$	0	0·5
	$1{\cdot}3\times10^5$	0	3
	$6{\cdot}5\times10^5$	4	11
III	$0{\cdot}3\times10^5$	0	0
	$1{\cdot}6\times10^5$	0·5	1·5
	$7{\cdot}9\times10^5$	9·5	2·7
IV	$1{\cdot}6\times10^5$		5
	$6{\cdot}8\times10^5$	7	15

It is clear from the results shown in Table 1 that immunization of this fowl failed to enhance the capacity of its white blood cells to produce foci on the CAM of either outbred embryos or embryos of the AA line.

Immunization of outbred fowls with tissue suspensions from outbred embryos.

In view of this result it seemed highly desirable to repeat the experiment by immunizing several additional fowls with preparations of embryonic tissues. However, sufficient inbred embryos for further experiments were not available. To overcome this difficulty it was decided to immunize outbred fowls with suspensions of splenic tissue from outbred embryos and to prepare each inoculum from as large a number of embryos as was practicable in order to introduce a wide variety of antigenic determinants into each immunizing dose. This decision was based on the assumption that the number of histocompatability antigens in fowls is of the same order as that found in mice (Gorer and Mikulska, 1960).

Since there is a gradual build up of adult antigens in the chick embryo as the embryonic age increases (Ebert, 1952) and since the existence of transient embryonic antigens has been demonstrated (Van Alten, 1959) it was decided to immunize individual birds with spleen suspensions derived from embryos at

different stages of development. The post-immunization bleeds were titrated in the same way. It was not, of course, possible to titrate pre- and post-immunization bleeds in parallel, but Szenberg and Warner (1962) have shown that titrations of successive bleeds from individual normal fowls yield a fairly consistent LML/focus ratio.

A comparison of the results of titrations of pre- and post-immunization bleeds from seven fowls immunized with spleen tissue from embryos of different ages is shown in Table 2. These results show that the immunization procedures employed have failed to bring about any significant changes in the LML/focus ratio. The LML/focus ratio of spleen suspensions from three of the immunized fowls was also determined and these figures are included in Table 2. As found in the case of normal fowls (Szenberg, Warner, Burnet and Lind, 1962) the spleen LML of immunized fowls are less efficient in producing foci on the CAM than the LML of the blood.

TABLE 2.

Effect of immunizing fowls with spleens from embryos of different ages on the ability of their circulating large lymphocytes (LML) to produce chorio-allantoic foci.

Fowl No.	Age of embryos from which immunizing antigen derived	LML/focus		
		Blood		Spleen
		Pre-immune	Immune	Immune
175	12 day	1400	1800	
131	12 day	900	1000 960	
116	16 day	2100	2300	19300
117	16 day	5750	2900	8300
174	17-19 day	1100	1400	
139	18 day	1500	1700 800 2000	
47	18 day	1800	1800 1300	3800

The immune status of the donor fowls.

The development of a positive direct Coombs' reaction in recipient chickens.

In order to see if the donor fowls had been adequately immunized a second set of experiments was carried out, in several instances, in parallel with the titration of the fowl blood on the CAM. The same blood sample which was used for titration on the membrane was inoculated intravenously into 18-day chick embryos which, after hatching, were tested for the development of a positive direct Coombs' reaction. It was thought probable that inoculation of blood from immune adult donors would result in an earlier development of a positive Coombs' test than inoculation of blood from those fowls prior to immunization. However, while this work was in progress Warner (1962) reported that normal chickens frequently develop a strongly positive Coombs' reaction during the first

TABLE 3.

Development of positive Coombs' reaction by the 3rd post hatching day among chickens inoculated intravenously on the 18th embryonic day with non-immune and immune adult fowl blood.

Adult fowl blood inoculum	No. chickens tested	Coombs' reaction		
		negative	weak to moderate	strong
Pre-immune	46	91 p.c.	9 p.c.	nil
From fowls immunized with 12 day embryonic spleens	36	86 p.c.	14 p.c.	nil
From fowls immunized with 16-18 day embryonic spleens	39	41 p.c.	24 p.c.	35 p.c.

week after hatching and that this is often maintained well into adult life. In view of Warner's findings it was clear that significance could only be attached to an enhancement in the numbers of chickens giving a positive Coombs' test during the first three days after hatching. Table 3 shows the numbers of chickens inoculated during embryonic life with both pre- and post-immunization adult fowl blood giving a positive direct Coombs' test by the third day after hatching. The chickens hatching from embryos inoculated with cells from adult fowls inoculated with 12-day embryo tissue show no significant difference from those inoculated with blood from the same fowls before immunization was begun. However, the proportion of both moderate and strong positives among chickens which had received blood from donors immunized with tissue from the older embryos is significantly higher.

In each case the immunization programme outlined under Methods was followed. Prior to immunization the fowls were bled into heparin (final concentration, 5 units per ml.) and a differential cell count was made on the blood. Dilutions of blood containing known numbers of large lymphocytes in the range $0{\cdot}2\text{-}0{\cdot}8 \times 10^5$ per $0{\cdot}1$ ml. were then inoculated onto 16-20 dropped CAM of 12-day outbred embryos for determination of CAM focal count and the LML/focus ratio.

Further experiments were carried out in which chickens were inoculated with immune blood on the 16th instead of the 18th embryonic day, but there was no evidence of an earlier development of positive Coombs' reactions in these groups. Still earlier inoculations led to a very high proportion of embryonic deaths prior to hatching.

The development of adult and embryonic fowl cell haemagglutinins in the immunized donor fowls.

Sera from certain of the immunized donor fowls were tested by the presence of adult and embryonic fowl cell haemagglutinins with the results shown in Table 4. Only those fowls inoculated with 18-day embryonic spleens gave a response in this test. Those inoculated with 12-day embryonic spleens were uniformly negative.

TABLE 4.

Haemagglutinin titres of normal and immunized adult fowls for adult and embryonic fowl red cells.

Fowls	Antigen	Haemagglutinin titres for cells from		
		Adult fowls	18 day embryos	12 day embryos
Normal fowls				
1	nil	< 2	< 2	< 2
2	nil	< 2	< 5	< 5
3	nil	< 2	< 5	< 5
4	nil	10	10	20
Immunized fowls				
131 Immune bleed I	12 day spleens	3	2	2
Immune bleed II		< 5	< 5	< 5
180 Preimmune	12 day spleens	< 5	< 5	< 5
Immune bleed I		< 2	< 2	< 2
139 Immune bleed I	18 day spleens	1600	220	200
47 Immune bleed I	18 day spleens	160	320	160

DISCUSSION.

These results provide no evidence that the efficiency of the large lymphocytes of adult fowls in producing foci on the chorio-allantois is enhanced by pre-immunization of the donor fowls with embryonic tissues. The apparent exception is in the case of fowl 117 (Table 2) where a twofold increase in efficiency was observed after immunization. In this instance, however, the pre-immune LML/focus ratio was well beyond the normal value of 2000 LML/focus (Szenberg and Warner, 1961) which might indicate some technical error; the post immune result was high but within the normal range. A twofold increase in lesion-producing capacity cannot be considered very significant in this system.

Szenberg, Warner, Burnet and Lind (1962) found that spleen LML were much less efficient than blood LML in producing chorio-allantoic foci. We have found that after immunization the spleen LML/focus ratio is still much higher than that found for blood LML from the same fowls, and is in the same range as that found for normal fowls. It was also noted that in immunized fowls the frequency of occurrence of plasma cells in the spleen suspensions was 4-5 times higher than in normal fowls.

There are two possible explanations for our results. Firstly, that the immunization procedure was inadequate, or secondly, that the whole large lymphocyte population of the normal, non-immunized fowl is fully active and that this activity cannot be further enhanced by immunization. As to the first explanation it would appear both from the development of haemagglutinins in the donor birds following immunization and from the results of Coombs' tests on chickens inoculated during embryonic life with cells from immunized birds that fowls inoculated with 16-18-day embryonic tissue show an immune response. However, material from 12-day embryos does not appear to provide an adequate

antigenic stimulus. This latter finding is in agreement with that of Mun, Kosin and Sato (1959). The concept of the full activity of the large lymphocytes of unstimulated birds is supported by the fact that in some cases it has been possible to produce a single focus with as few as 40 large lymphocytes (Szenberg and Warner, 1961).

From these results and from previous experiments it has been concluded (Szenberg, Warner, Burnet and Lind, 1962) that the clonal selection theory of antibody production (Burnet, 1959) does not apply without modification to the Simonsen phenomenon.

The present results do not allow any presentation of an alternative hypothesis since it must be emphasized that no evidence has yet been obtained of the number or nature of histocompatibility antigens in the fowl, nor is it known whether there is any relation between haemagglutinating antibodies and homograft immunity in this species. All that can be said is that prolonged immunization procedures with material which must have contained a number of histocompatibility antigens foreign to the treated donors, resulted in the development of haemagglutinating antibodies, but showed no change of any significance in the LML/focus ratio. Investigations are in progress by which it is hoped to determine the approximate number of histocompatibility antigens involved in the CAM reactions and the significance, at the moment problematical, of the increased incidence of apparent Coombs' reactions in the present work.

Acknowledgments. The authors are indebted to Sir Macfarlane Burnet, O.M., F.R.S., for helpful discussions and criticism in the course of this work, and to Miss Janet Irwin and Mrs. Jill Bray for technical assistance.

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A METHOD FOR THE MEASUREMENT OF TOTAL NITROGEN IN MICROGRAM AMOUNTS

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(*Accepted for publication 3rd November, 1961.*)

SUMMARY.

A method is described for the measurement of total nitrogen in the range 0·5-25 μg. It can be employed routinely, and is readily adaptable to different requirements. The method involves a modified Kjeldahl digestion, and the ammonia is distilled in Conway's micro-diffusion vessels. It is accurate and simple in operation.

INTRODUCTION.

During a recent study on nitrogen-fixation using *Azotobacter*, it was necessary to follow nitrogen fixation in bacterial cultures of very low density. Warburg micro-respirometer flasks were used to grow the organism under conditions of controlled temperature and atmosphere (Parker and Scutt, 1960). The bacterial nitrogen present in each flask was 3-4 μg. at the commencement of the experiments, increasing (according to treatment) to 8-15 μg. N after three hours' growth. An accurate routine method of total nitrogen analysis was needed to determine changes in this bacterial nitrogen.

The method of Lowry *et al.* (1951) seemed promising. In this, protein is measured by the Folin phenol reagent, which gives a blue colour with certain aromatic amino acids. A short digestion of the bacterial cells with alkali is necessary to liberate the cell components. However, this digestion produced, from the glucose or sucrose of the bacterial growth medium, compounds which also gave the blue colour with the phenol reagent.

Russell (1944) estimated ammonia nitrogen at the microgram level using the phenol-hypochlorite reaction. We obtained good colour development with this method, but we were unable to get consistent results.

It was decided to measure the bacterial nitrogen directly, although the amounts of nitrogen involved were very small. There was also the problem of removing the water from the bacterial sample before effective digestion temperatures could be achieved. Conway's book (1950) on micro-diffusion analysis, and a paper by McKenzie and Wallace (1954) on the temperature-time relationship in Kjeldahl digestion, were drawn upon heavily in the development of a satisfactory method.

Materials and Methods.

Reagents and apparatus—

Boiling mixtures: (i) Low temperature mixture. This is to boil off the water from the sample; its boiling point can be adjusted to give rapid removal of the water without bumping and loss of contents. For 1 ml. cultures we used a ratio of H_2SO_4/H_2O of 1:2 (by volume), the actual quantities being 175 ml. acid added to 350 ml. of water in a 1-litre beaker.

(ii) High temperature mixture. 100 gm. Na_2SO_4 in 100 ml. conc. H_2SO_4.

Digestion mixtures: (i) One volume of pure sulphuric acid in 1 volume of distilled water.

(ii) 0·5 p.c. selenium solution. To prepare this solution boil 5·0 gm. selenium in 100 ml. conc. H_2SO_4 until clear to give a stock solution. Add one part stock solution to nine parts 20 p.c. Na_2SO_4 solution for use.

Reagents used in Conway units: Boric acid, prepared as advised by Conway (1950), p. 96 (but using 0·2 p.c. boric acid); HCl, 0·004 N; NaOH, 40 p.c.; paraffin, hard white vaseline of high purity (Cheesebrough Manufacturing Co.).

Apparatus: (i) Conway No. 1 (standard) units; (ii) 0·8 ml. and 1·0 ml. pipettes (Conway, 1950); (iii) Pasteur pipettes; (iv) 190 mm. x 16 mm. Pyrex test tubes with glass caps; (v) one 1-litre Pyrex beaker cut down to 10 cm. to take boiling mixture (ii); (vi) one 1-litre Pyrex beaker to take boiling mixture (i); (vii) quartz sand.

Removal and transfer of cultures:

(i) Wipe vaseline from neck of Warburg flasks.

(ii) Add 0·3 ml. digestion mixture (1).

(iii) Swill flasks with circular motion and remove contents with Pasteur pipette into digestion tubes.

(iv) Rinse flasks with 0·3 ml. distilled water, taking this also into digestion tubes.

Digestion:

(i) Add 0·3 ml. digestion mixture (ii) to tubes. This can be added earlier to all tubes (including controls, which must also receive 0·3 ml. of digestion mixture (i));

(ii) Boil off excess water by placing tubes (without caps) in deep bath containing boiling mixture (i) and boil them until frothing has stopped;

(iii) Remove tubes into shallow bath containing boiling mixture (ii); add small pinch of quartz sand to bath to prevent bumping. Place the glass caps over the tubes and heat strongly. Allow one hour's vigorous reflux after clearing; the final temperature of the bath should be at least 360° C. Cool, add 2 ml. glass distilled water to all tubes.

Diffusion of ammonia:

(i) Place Conway units on a slight incline by resting on 2 mm. steel slats, so that the acid remains on one side of the unit. To the centre wells (their rims should have been lightly greased beforehand) add 1·0 ml. 0·2 p.c. boric acid solution. From each tube take two aliquots with 0·8 ml. pipette into the units. Take care not to splash the acid or touch the walls of the unit with the tip of the pipette.

(ii) Conway's glass cover-plates are greased with vaseline and placed on the units, leaving about ¼-inch gap on upper side.

(iii) Through this gap add 1 ml. 40 p.c. NaOH without splashing. Close the lid and see that the grease seal is intact all around the rim of the unit.

(iv) Mix contents gently and place on the rotor (see Fig. 1) or oscillatory table (Conway, 1950). Allow two hours' diffusion time on either machine.

(v) Titrate with 0·004 N HCl to standard colour, using control blanks of reagents and medium. CO_2-free air blown over the titration surface improves the results by removing CO_2-laden air from the operator's breath.

EXPERIMENTAL.

Conway (1950), p. 131, suggested that water might be removed from a digestion mixture by placing the digestion vessels in an oven at 105° C. overnight. In our experiments heavy losses of nitrogen occurred when the water was removed in this way (Table 1). In washing the diffusion apparatus, the methods described by Conway were quite inadequate with our glassware.

TABLE 1.

Recovery of nitrogen in micrograms from 2 ml. of an Azotobacter *culture in a 1 p.c. glucose-salts medium. Water removed in (i) oven at 105° C. (ii) boiling bath.*

Oven at 105° C.	Boiling bath
4·5	6·3
4·8	6·5
5·4	6·5

After careful scrubbing of the units in a neutral detergent (Lissapol N300), they were rinsed under hot water and autoclaved in distilled water for one hour at 5 p.s.i. pressure. The water was tested for pH after autoclaving, and the units were "passed" if the pH was close to neutral. We found the autoclaving procedure essential to obtain reproducible results.

Removal of water from the sample. This was achieved by immersing the digestion tubes with their contents in a deep bath of sulphuric acid and water in a fume cupboard. The concentration of sulphuric acid used is determined by the amount of water in the sample and the need to avoid bumping within the tubes. About half the length of the tubes should remain above the boiling mixture to allow for some reflux. By vigorous boiling the preliminary bath can be made to take the digestion past the frothing stage if this is likely to be troublesome during the true digestion. The time required to boil off the water and digest the contents past the frothing stage was about 70 minutes.

Digestion. This is carried out in the same tubes, in a shallow boiling bath which allows sufficient refluxing surface to prevent excessive loss of acid from the tubes. A one-litre beaker cut down to 10 cm. in height was used, so that the digestion tubes had about 7-8 cm. of their length open to the cooling effect of the forced draught of the fume cupboard. The mixture in the boiling bath has a higher boiling point initially than the digestion mixture in the tubes, so that a more vigorous reflux takes place within them. The presence of sodium sulphate in the latter mixtures ensures that some sulphuric acid remains in the bottom of the tube, because the boiling points of the mixtures inside and outside the tubes eventually become equal. According to the results of McKenzie and Wallace (1954), at a temperature of 360° C. complete recovery can be expected after one hour's digestion. We obtained complete recovery from tryptophane (see Table 2) in one hour's digestion after clearing, when the temperature in

the digestion tubes had just reached 360° C. With nicotinic acid, however, two hours' digestion after clearing was needed, and the temperature had reached 380° C.

TABLE 2.

Recovery of N in micrograms from 10 micrograms tryptophane-N at 30, 60 and 90 minutes after clearing.

30 minutes	60 minutes	90 minutes
9·4	9·8	9·8
9·3	9·9	10·2
9·5	10·1	9·9

On cooling, 2 ml. of distilled water are added to the tubes, the total volume of the digest being taken as 2·1 ml. Where the temperature of digestion is the same for all digests (as it is in the bath type digestion) the error of this method is very small (Conway, p. 142).

Microdiffusion. Duplicate distillations can be made from each digest if desired. Diffusion of ammonia from the Conway units can be hastened by agitation on an oscillatory table (Conway, p. 21). A circular table which revolves on an incline (see Fig. 1) was also found to be satisfactory, cheap to construct, and able to carry a heavy load of Conway units.

Table 3 gives a comparison of ammonia diffusion on the two different types of agitating machines. The recoveries do not differ significantly.

TABLE 3.

Recovery of nitrogen from 10 micrograms N as ammonium sulphate on (i) oscillatory table and (ii) rotor with different times.

Time in hours	Oscillatory table		Rotor	
2	9·74	9·74	9·80	9·80
3	9·91	9·85	9·91	9·97
4	9·85	9·91	9·91	9·85

Titration. The end-point of the titration can be made more sensitive by reducing the boric acid concentration (Yuen and Pollard, 1953). At higher levels of nitrogen better results were obtained with 0·2 p.c. than with 0·1 p.c. boric acid (see Table 4), apparently because 0·1 p.c. boric acid did not absorb the ammonia as readily.

Precautions. Care must be taken to prevent contamination of apparatus or reagents by ammonia in the atmosphere, or by alkaline dust. To this end our laboratory is routinely sprayed with an emulsion of light paraffin oil in water before conducting the diffusion part of the experiment. The boric acid method

TABLE 4.

Absorption of ammonia by boric acid solutions at 0·2 and 0·1 p.c. concentration.

	Initial N in micrograms				
	5·0	10·0	15·0	20·0	25·0
Nitrogen recovered using:					
0·2 p.c. boric acid	4·8	9·6	14·4	18·9	24·2
0·1 p.c. boric acid	4·6	9·0	13·6	17·3	20·6

Fig. 1.

of absorption has some very real advantages over the acid method, because traces of alkali or acid present on the apparatus or in the atmosphere are readily detected by colour changes which occur in the boric acid-indicator solution in the centre wells. Thus, both pipe and cigarette smoking visitors, new paint and the calcareous dust arising from a hole which was being bored in wall plaster, have been implicated during our experiments. The smoke from the burning end of a cigarette or pipe contains large amounts of ammonia. The plaster dust came from a room below our laboratory and at least 80 feet away.

RESULTS.

Using the method described, we have obtained considerable precision in routine estimations of total nitrogen in Warburg cultures. Table 5 gives the results of an experiment designed to test the effect of digestion time on recovery of nitrogen from bacterial cultures.

TABLE 5.

Recovery of nitrogen in micrograms from bacterial cultures at 30, 60 and 90 minutes' digestion after clearing.

30 minutes	60 minutes	90 minutes
5·1	5·7	5·6
5·1	5·6	5·7
5·7	5·7	5·7

It can be seen that one hour's digestion time after clearing is sufficient for full recovery (compare Table 2).

The reliability of a method is very important in comparative work. Table 6 sets out the results from an experiment in which 12 aliquots of a culture of *Azotobacter* were analysed for total nitrogen by the method described.

TABLE 6.

Duplicate titrations in 0·004 N HCl from twelve 1 ml. samples of a bacterial culture (0·8 ml. aliquots from 2·1 ml. of digest were pipetted into Conway units).

Tube	1	2	3	4	5	6
	0·035 0·036	0·034 0·035	0·038 0·037	0·035 0·035	0·036 0·036	0·036 0·036
Tube	7	8	9	10	11	12
	0·036 0·036	0·037 0·037	0·036 0·035	0·037 0·036	0·036 0·034	0·036 0·035

S.D. = 0·00089 ml. 0·004 N HCl (equivalent to 0·13 μg. nitrogen).

The method can be readily adapted, by appropriate alteration of the reagent concentrations, to measure higher or lower ranges of nitrogen than were covered in these experiments. For solid substances the low temperature boiling bath can be omitted.

The method has been used routinely in our laboratory by relatively untrained assistants, and has given consistently good results.

Acknowledgments. I am grateful to Mr. N. S. Stenhouse, of the Division of Mathematical Statistics, C.S.I.R.O., for the statistical analysis of the data.

The work was done with the aid of financial assistance from the Rural Credits Development Fund of the Reserve Bank of Australia.

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STUDIES OF A VIRUS ISOLATED FROM A WILD HOUSE MOUSE, *MUS MUSCULUS*, AND PRODUCING SPLENOMEGALY AND LYMPH NODE ENLARGEMENT IN MICE

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(*Accepted for publication 16th October, 1961.*)

SUMMARY.

A virus (WM1 strain) was isolated from a wild house mouse, *Mus musculus*. It was infective for suckling and weaned mice inoculated intraperitoneally, but not for laboratory *Rattus norvegicus*, guinea-pigs, rabbits or chick embryos.

A prolonged chronic infection resulted, with normal blood picture, persistent viraemia, moderate splenomegaly and general enlargement of lymph nodes, which were sometimes massive. Most infected mice died within 11½ months.

Microscopically, the splenomegaly was seen to be due to hyperplasia of both red and white pulp, while enlargement of lymph nodes was essentially due to medullary hyperplasia, with plasma cells prominent. Four of 79 infected weaned mice examined at random within 11½ months had developed neoplasms of the lymph nodes, while none was seen in any of the 87 control mice examined in the same period. However, two of 17 control mice had neoplasms of lymph nodes at 18 months.

The virus passed through Gradocol membranes with average pore diameter of 320 mμ. It was sensitive to ether, formalin, and heating at 56° C. for 30 minutes, but remained viable at −60° C. and after lyophilization, and was resistant to 5 p.c. ethanol at 4° C. for 24 hours.

INTRODUCTION.

During a study of the rôle of wild animals as possible reservoirs of human pathogens in Queensland, an agent which proved to be experimentally transmissible in mice was isolated from a wild house mouse. Studies made on the nature of the agent and of experimental infection of mice are described.

Materials and Methods.

The mice used in this work were random-bred in the Institute and have been described previously (Derrick *et al.*, 1954). No other mouse strain is maintained. Weaned mice were inoculated when three weeks old, and suckling mice within 48 hours of birth. The inoculum for routine intraperitoneal (IP) passage in weaned mice was 0·5 ml. of the supernate of a crude saline suspension of liver and spleen which was allowed to settle for a few minutes. Intracerebral (IC) inoculation of weaned mice was made under ether using 0·03 ml.

Suckling mice received 0·03 ml. IP. Control mice were inoculated with suspensions prepared in a similar manner from the tissues of normal mice.

Spleen and lymph node sizes were recorded as the product of length and breadth (in mm.). For convenience in analysis a splenic product of 100 or more was termed splenomegaly. Spleens or lymph nodes were regarded as significantly enlarged when they exceeded the sum of the mean and twice the standard deviation ($\bar{x} + 2SD$) of the organs of the control group examined at the same time. In certain experiments the mean sizes of spleen and lymph nodes of infected and control groups of mice were compared by calculation of the standard error of the difference of the means.

For titration of infectivity of mouse tissues, tenfold dilutions up to 10^{-7} were prepared in saline from the uncentrifuged supernate of a 10^{-1} saline suspension, and 0·5 ml. was inoculated into each of a group of mice (usually six). Quantitative work was made difficult by the variable nature of the response of mice to infection. Inoculation of dilutions as low as 10^{-1} rarely resulted in an incidence of splenomegaly of 100 p.c. (see Table 1). However, as an attempt at quantitation was desirable, the ID_{50} of the inoculum was calculated from the incidence of splenomegaly in infected mice 28 days after inoculation, using the method of Reed and Muench (1938). Although this method of quantitation obviously lacked sensitivity, it served to indicate approximate levels of virus concentration.

Gradocol membranes were obtained from the Commonwealth Serum Laboratories, Melbourne, and from the Wright-Fleming Institute, St. Mary's Hospital, London. Filtration was carried out at 5-7 pounds per square inch positive air pressure, and each membrane was later checked by filtering a viable suspension of bacteria (usually *Serratia marcescens*) and culturing part of the filtrate.

EXPERIMENTAL.

Isolation, maintenance and general history of the virus.

A wild house mouse, *Mus musculus*, was captured in Brisbane, and autopsied on 3rd January, 1952. It was a male weighing 10 gm. and no abnormalities were observed at autopsy. A saline suspension of liver and spleen was prepared, inoculated into mice, and serial IP passages made. It was evident from the incidence of splenomegaly in the first seven passages that an agent producing splenomegaly was being transmitted. It was named the Wild Mouse 1 (WM1) strain after the mouse of origin. Several lines were subsequently maintained in serial passages, usually at 14-21-day intervals when the only indication of its presence was the occurrence of moderate splenomegaly.

Three other organisms of rodents which may cause chronic splenomegaly in mice are *Haemobartonella muris*, *Eperythrozoon coccoides* and *Spirillum minus*, but none of these was present in the blood of infected splenectomized mice.

At times, passages were made at longer intervals, and in some of these mice other abnormalities were seen. Definite enlargement of lymph nodes was first observed in a mouse of the 17th passage that had been held for five months before autopsy. In later passages, lymph nodes of occasional mice were considered to be slightly enlarged and it was found that the mean size of the inguinal lymph node of 11 mice of the 32nd passage examined nine months after inoculation was significantly greater than that of 17 control mice.

Inoculation by the IC or subcutaneous routes appeared to be as effective as IP, while intranasal inoculation was less so. Ingestion of virus was quite ineffective in producing the disease.

Experimental infection of weaned mice.

It was decided to investigate in greater detail the suggestion that the WM1 strain was capable of causing recognizable disease of the reticular tissue, partly with the hope that a more specific indicator of infection than splenomegaly would be found.

Mice were inoculated IP with a crude 10^{-1} saline suspension of liver and spleen of infected mice, and control mice received a suspension of liver and spleen of normal mice. Groups of six were examined at weekly intervals up to four weeks after inoculation, and from then on groups of up to 12 mice were examined at 3, 6, 9 and 11½ months after inoculation. The final observations on the controls were made at 18 months. Observations made included erythrocyte and leukocyte counts, differential leukocyte counts, and spleen and lymph node sizes. A pool of liver and spleen of each mouse (both infected and control) was subinoculated to test for the presence of virus, and in addition, an infected male and an infected female mouse were studied in more detail by titration of certain organs. Tissues were taken for histological examination.

Erythrocyte and leukocyte counts. Infection of mice with the virus appeared to cause a significant increase in leukocytes at seven days followed by a significant decrease at 14 days. Thereafter, there was no consistent deviation from normal levels.

Splenomegaly and lymphadenopathy. Observations on the sizes of spleens and lymph nodes of mice at various periods after infection with the virus, and also of the uninfected control mice, are recorded in Table 1.

From 14 days after infection there was a significant increase in the mean spleen size of infected mice of all but two groups compared with the controls. The mean size was higher at three months than it had been at 1-4 weeks, and remained at that level (about 140-150) until nine months after infection. At 11½ months the mean was slightly lower, but there were fewer mice than usual in these groups. The incidence of splenomegaly was consistently higher in the groups of infected mice than in the control groups examined at the same time after inoculation. In the period up to 11½ months after inoculation, the total incidence of splenomegaly in 174 infected mice (including those that were killed and those that died) was 82 p.c., while in 99 control mice it was 30 p.c. A higher degree of splenomegaly (i.e. spleen size of 200 or more) was recorded in 16 p.c. of infected and in 3 p.c. of control mice.

It is apparent from Table 1 that the mean lymph node sizes of groups of infected mice were often higher than those of control groups, and this was shown most clearly with the lumbar nodes. In the 12 groups of mice examined between one week and 11½ months after inoculation, a mean lymph node size significantly higher than that of the controls was recorded for the lumbar in

TABLE 1.

Mean values of spleen and lymph node size, and incidence of enlargement in infected and control mice.

Time since inoculation	Sex	Inoculum	Spleen		Lymph nodes				
			Incidence of splenomegaly	Mean size	Inguinal mean size	Lumbar mean size	Mesenteric mean size	Incidence of significantly enlarged lumbar lymph nodes	Incidence of greatly enlarged lymph nodes[1]
7 days	♂	WM1	3/6	106	6·2*	3·0*	43	3/6	0/6
		Control	2/6	81	3·3	1·5	31	0/6	0/6
14 days	♂	WM1	6/6	109*	7·8*	4·1*	50	5/6*	0/6
		Control	1/6	82	3·3	1·8	47	0/6	0/6
21 days	♂	WM1	4/6	103*	7·1*	4·6*	59	4/6	0/6
		Control	0/6	84	3·9	1·5	61	0/6	0/6
28 days	♂	WM1	5/6	115	7·6*	3·9*	70	4/6	0/6
		Control	3/6	93	3·8	1·6	59	0/6	0/6
3 months	♂	WM1	11/12	147*	14·0	9·1	66·3	5/12	1/12
		Control	1/7	93	11·0	7·1	55·2	0/7	0/7
	♀	WM1	12/12	154*	14·5*	15·5*	64·0*	6/12*	1/12
		Control	4/9	90	7·1	7·8	52·4	0/9	0/9
6 months	♂	WM1	10/11	146*	13·1*	12·4*	46·8	6/11*	1/11
		Control	2/10	87	8·8	7·0	51·5	0/10	0/10
	♀	WM1	11/12	144*	14·5*	11·5*	52	5/12	1/12
		Control	3/12	88	8·0	6·9	52	1/12	0/12
9 months	♂	WM1	10/11	148*	8·9	13·9*	48·5	9/11*	1/11
		Control	1/11	75	6·3	3·9	43·0	0/11	0/11
	♀	WM1	11/12	146*	19·3	20·2*	57·0*	10/12*	3/12
		Control	4/12	78	4·8	2·7	33·5	1/12	0/12
11½ months	♂	WM1	3/7	114	8·4	11·5*	39·8	2/7	0/7
		Control	3/12	85	7·7	5·7	52·9	1/12	0/12
	♀	WM1	3/4	129*	10·2	12·7*	43·5	3/4*	0/4
		Control	3/13	95	6·2	4·6	31·9	0/13	0/13
18 months	♂	Control	2/14	83	5·3	7·2	44	1/14	2/14
	♀	Control	2/3	107	8·2	15·5	33	0/3	1/3

* Indicates values significantly different from those of controls.

[1] Includes those mice with nodes (excluding the mesenteric) with a product of 40 or more.

11 groups, for the inguinal in seven, and for the mesenteric in two. The incidence of significantly enlarged lumbar lymph nodes was consistently higher in infected than in control groups. In the latter only four significantly enlarged nodes were recorded, and those were in mice examined 6-18 months after inoculation. During the period up to 11½ months after inoculation, the total incidence of significantly enlarged lymph nodes in 174 infected mice (including those that were killed and those that died) was 71 p.c. while in the 99 controls it was 6 p.c. Greatly enlarged lymph nodes (see footnote of Table 1 for definition) were present in 35 (20 p.c.) of the 174 infected mice examined within 11½ months of inoculation, but none was observed in the 99 controls. However, in 2/17 of the control mice examined 18 months after inoculation greatly enlarged lymph nodes were seen.

In general, slight or moderate lymph node enlargement involved most major nodes. On the other hand, more extreme enlargement usually involved one or two nodes only and this was noted in 26 of the 35 mice with greatly enlarged nodes (74 p.c.), while the rest showed great enlargement of four or more nodes (Fig. 1). In these 35 mice, great enlargement was most common in inguinal, lumbar and cervical nodes (12/35, 13/35, and 13/35, each about 37 p.c.), while similar enlargement of the mesenteric (5) and axillary (4) lymph nodes was recorded less often. Thymus was greatly enlarged in six of the 35 mice.

Fig. 1. Mouse autopsied five months after inoculation with WM1 virus, showing generalized lymph node enlargement and splenomegaly.

Histopathology. Study was made of various organs of 79 infected weaned mice and of 104 controls killed between three and 18 months after inoculation. Lymph nodes (usually inguinal, lumbar, cervical, mesenteric, and any other showing enlargement), spleen, bone marrow, liver, kidney, salivary glands, lung, heart, skeletal muscle, brain and spinal cord were included. The only organs showing changes that could be ascribed to the infection

* Indicates values significantly different from those of controls.
† Includes those mice with nodes (excluding the mesenteric) with a product of 40 or more.

were the lymph nodes and spleen. About 57 p.c. of these 79 mice had lymph nodes that were significantly enlarged (see Table 1), while in the remainder the size varied from just below a significant level to apparently normal. In the majority of mice the enlargement was hyperplastic, but in four it was neoplastic (Table 2). All four were males. The two types of neoplasm seen were lymphocytic neoplasm and reticulum-cell neoplasm Type B (Hodgkins-like lesion). The terminology of Dunn (1954) has been followed in the present work.

Two mice had lymph nodes with lymphocytic neoplasm. There was complete loss of normal structure, and the nodes consisted of masses of lymphocytes, with increased mitoses. Infiltration of the capsule by lymphocytes was commonly seen, and in one mouse there was extensive infiltration of salivary glands (Fig. 2). In the other three mice the neoplasm were essentially localized.

The reticulum-cell neoplasm Type B was diagnosed in lymph nodes of two mice, and was characterized by almost complete loss of nodular structure (Fig. 3), the presence of a mixture of cells including reticulum cells, lympho-

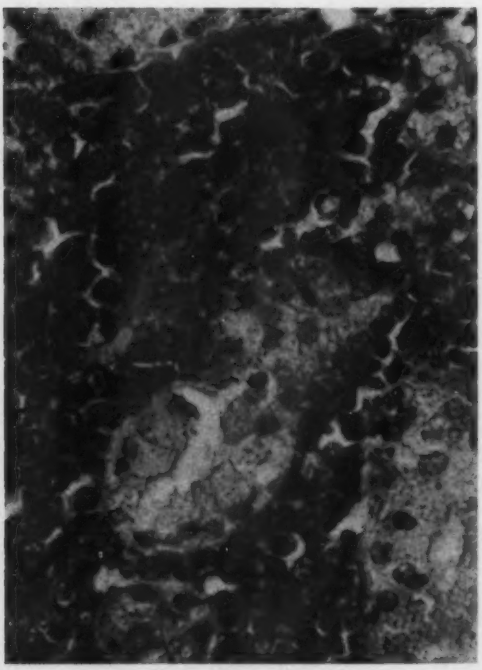

Fig. 2. Salivary gland of mouse MQ17,785 three months after inoculation. Lymphocytic neoplasm with extensive infiltration of abnormal lymphocytes among scattered remaining ducts. H and E. x 320.

Fig. 3. Portion of cortex of lymph node of mouse MQ17,771 three months after inoculation. Reticulum-cell neoplasm Type B showing loss of nodular structure and the presence of a mixture of cells. H and E. x 96.

cytes and plasma cells, and a marked increase in reticulum cells, not only in the intermediate zone and medulla, but in the cortical region as well (Fig. 4). There was little or no phagocytosis, and no infiltration of the capsule.

The mice examined histologically included twelve with greatly enlarged lymph nodes. Three of the neoplasms were found in these twelve; the fourth came from a mouse with moderately enlarged lymph nodes.

It should be emphasized that no neoplasms of lymph nodes were present in any of the 87 control mice examined at various intervals up to 11½ months after inoculation, and the three lymph nodes that were significantly enlarged were hyperplastic. However, 2/17 control mice examined 18 months after inoculation showed lymphocytic neoplasms, one involving thymus and the other the lymph nodes.

The hyperplastic reaction in lymph nodes had two forms. In the simplest form the normal structure of the node remained (Fig. 5), with lymphocytes

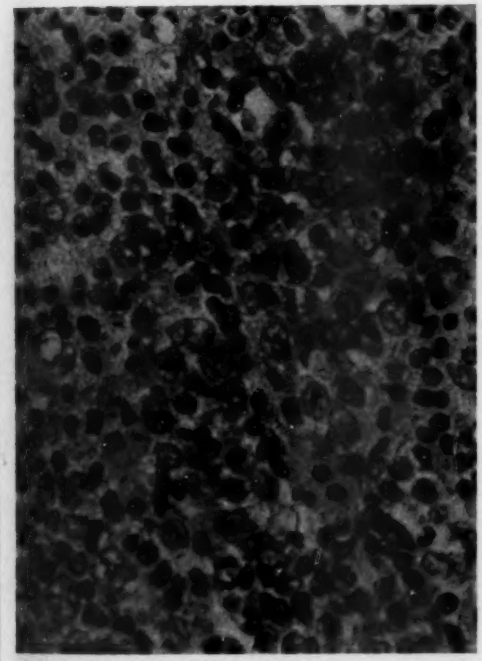

Fig. 4. Higher power of the same lymph node, MQ17,771, showing types of cells present. Reticulum cells are increased in number. Lymphocytes and occasional plasma cells are also present. H and E. x 320.

Fig. 5. Enlarged lymph node of mouse MQ17,824 6½ months after inoculation. This is a typical example of the hyperplasia seen, with the normal structure of the node remaining. H and E. x 28.

predominating in the cortex and with sometimes an increase in the number of nodules. The increase in size was essentially due to increase in the number of plasma cells and lymphocytes in the intermediate zone and medulla. In older mice (i.e. six months or more), the nodules were usually of reaction centre type, with reticulum cells predominating and some phagocytosis, while the sinusoids were usually dilated and prominent. Reticulum cells were more numerous in the medulla of these mice than in those of the three months' series. In the other form the nodular structure of the node was almost completely obliterated (Fig. 6), and the node consisted mainly of a mixture of lymphocytes and plasma cells, while reticulum cells were not prominent. These lymph node changes were considered to indicate infectious hyperplasia.

The enlargement of the spleen of infected mice was due to hyperplasia in all mice, and involved both the white and the red pulp, although the normal follicular structure remained (Fig. 7). The features of this general splenic hyperplasia were usually an increase in the size of the nodules, which commonly had

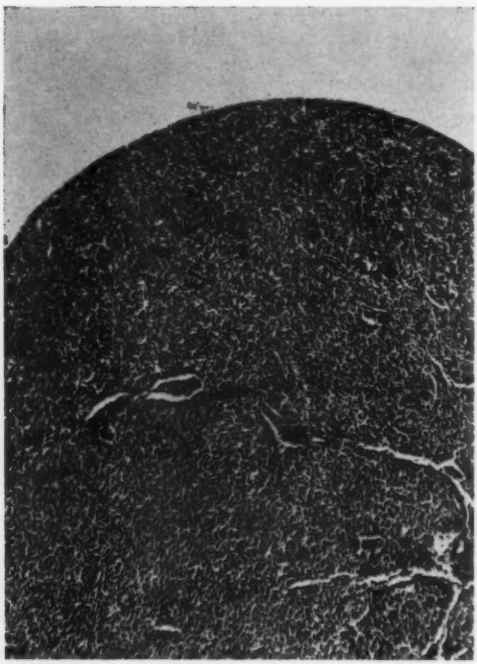

Fig. 6. Enlarged lymph node of mouse MQ17,856 9½ months after inoculation. This type of hyperplasia was less commonly seen. Nodules were absent completely. H and E. x 28.

Fig. 7. Enlarged spleen of mouse MQ17,838 6½ months after inoculation. It is hyperplastic, but normal structure remains. H and E. x 28.

reaction centres, and an increase in the number of reticulum cells in the white pulp. The latter was sometimes quite marked, particularly after six months. The red pulp also increased, but remained normal in appearance. These changes in the spleen were also considered to be those of an infectious hyperplasia.

Certain non-specific lesions were observed in both infected and control mice, and included small foci (usually periportal) of lymphoid or occasionally myeloid cells in liver, and small perivascular lymphoid foci in kidney and salivary glands. Also common was a pneumonia characterized by accumulation of plasma cells and lymphocytes about bronchioles and blood vessels and of polymorphs in the

TABLE 2.

Incidence of neoplasia in the lymph nodes of infected weaned mice killed at various times.

Months after inoculation	Sex	Proportion of mice with reticulum-cell neoplasm type B	Proportion of mice with lymphocytic neoplasm	Total proportion of mice with neoplasia
3	♂ ♀	1/10 0/10	1/10 0/10	2/10 0/10
6	♂ ♀	0/12 0/12	1/12 0/12	1/12 0/12
9	♂ ♀	1/12 0/12	0/12 0/12	1/12 0/12
11½	♂ ♀	0/7 0/4	0/7 0/4	0/7 0/4
Total		2/79	2/79	4/79 (5 p.c.)

TABLE 3.

Concentration of virus in mouse tissues at various intervals after inoculation.

Period since inoculation	Log ID_{50} (minus signs omitted)			
	Blood	Liver	Spleen	Lymph nodes
7 days	2·9 4·4	5·6 3·9	4·2 4·7	3·7 5·0
14 days	4·7 5·1	5·0 4·3	5·5 6·0	5·1 5·5
21 days	4·0 3·4	4·6 4·3	5·0 5·5	3·8 6·0
28 days	4·2 4·1	5·0 4·0	5·3 4·3	5·0 3·7
3 months	4·5 3·3	4·3 3·0	3·8 4·2	Not tested
6 months	4·5 4·5	5·0 4·3	4·3 6·3	Not tested
9 months	3·0 3·8	4·3 3·8	4·6 3·8	Not tested
11½ months	4·5 4·0	4·6 4·6	5·0 4·6	5·1 5·7

lumen of the bronchioles. In some older mice there was consolidation of alveoli with infiltration by polymorphs. The causes of these lesions are unknown at present.

Persistence of virus. Virus was recovered (as indicated by production of splenomegaly in subinoculated mice) from the pool of the liver and spleen of each of the 105 infected mice tested between seven days and 11½ months. The results of titrations of certain tissues of two infected mice at each interval after inoculation are shown in Table 3. The ID_{50} were calculated by the Reed-Muench method from the incidence of splenomegaly. Within seven days of inoculation, the ID_{50} of blood and other tissues tested had reached a level of the order of 10^{-4} which was maintained for the duration of infection. This technique did not show any marked difference between the concentration of virus in blood, liver, spleen or lymph nodes. Other studies have amply confirmed this long persistence of virus.

No transmissible agent producing splenomegaly was isolated from any of the 137 control mice individually tested by subinoculation, at periods from seven days to 18 months after inoculation, including the two with lymphocytic neoplasms. Splenomegaly occurred in some of the subinoculated mice, but on further passage it proved to be not transmissible.

Mortality. The main aim of the long-term experiment was to study the progress of the infection in mice, and for this purpose 89 of the 227 infected mice and 119 of the 230 controls were killed for the studies already described. The majority of the mice were taken at random and the remainder were left for observation. From the records of the dates of death of these mice, the percentage of mice dead at five-week intervals was calculated and is shown in Fig. 8. Allowance was made for the mice that were killed.

The mortality rate in infected mice was approximately twice that of the controls, and this became apparent 15 weeks after inoculation. Ninety-two p.c. of the infected mice died within 11½ months, at which time the remaining 11 were killed for examination. The mortality in the controls was 48 p.c. at 11½ months, and 86 p.c. at 18 months when the remaining 17 mice were killed. The mode of action of the virus in increasing the rate was not determined as often no apparent cause of death was detected. Mechanical interference with other organs by enlargement of lymph nodes may have been an important contributing factor. This was obvious in some mice, especially with the cervical and mediastinal nodes.

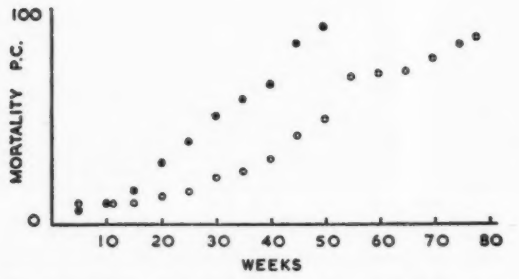

Fig. 8. Mortality in infected (●) and control (○) mice.

TABLE 4.

Common macroscopic abnormalities in dead mice (excluding those too decomposed for detailed study) up to 11½ months after inoculation.

Inoculum	Sex	No. of mice examined	No. with pneumonia	No. with visceral abscesses	No. with greatly enlarged lymph nodes	No. with spleens of 200 or more
CONTROL	♂	14	9	3	0	0
	♀	13	12	2	0	0
	Total	27	21 (77 p.c.)	5 (18 p.c.)	0	0
WM1	♂	35	15	3	5	8
	♀	42	26	7	15	8
	Total	77	41 (53 p.c.)	10 (13 p.c.)	20 (26 p.c.)	16 (20 p.c.)

The major macroscopic abnormalities seen at autopsy in mice that were closely studied are recorded in Table 4. Some mice showed more than one abnormality. Pneumonia was the most common disease observed, and contributed significantly to the mortality in both infected and control mice, although the incidence was slightly lower in the former. The mortality associated with abscesses was similar in both groups and was usually due to *Corynebacterium murium* which is present in the mouse colony.

Experimental infection of suckling mice.

The infection in mice inoculated within 48 hours of birth was similar in nature to that already described in weaned mice. The only difference observed (Table 5) was that over the first 11½ months the incidence of greatly enlarged lymph nodes was significantly higher (34 p.c.) in the mice inoculated during suckling than in those inoculated at weaning (20 p.c.). Suckling female mice showed the highest incidence of greatly enlarged lymph nodes (46 p.c.). The incidence of neoplasia observed in these mice up to 11½ months after inoculation was 3/50 (Table 6), and is comparable to the incidence observed in weaned

TABLE 5.

Total incidence of greatly enlarged lymph nodes in infected weaned and suckling mice over 11½ months.

Sex	Age at inoculation	Incidence	X^2		
♂	<48 hours	14/59 (23 p.c.)	2·0	5·2	4·1
	3 weeks	11/84 (13 p.c.)			
♀	<48 hours	24/52 (46 p.c.)	4·8		
	3 weeks	24/90 (26 p.c.)			

TABLE 6.

Incidence of neoplasia in mice inoculated <48 hours after birth and killed at various times.

Months after inoculation	Sex	Proportion of mice with neoplasms
3	♂	0/5
	♀	0/6
5*	♂	0/1
	♀	1/5 (Lymphocytic neoplasm)
7	♂	0/6
	♀	0/6
10	♂	0/5
	♀	0/5
11½	♂	1/7 (Reticulum-cell neoplasm Type B)
	♀	1/3 (Reticulum-cell neoplasm)
Total		3/50 (6 p.c.)

* This group was selected because of obvious lymphadenopathy.

mice. The neoplasia observed in the female mouse examined 11½ months after inoculation was different from the other two types seen in these infected mice. Spleen and liver were mainly involved and lymph nodes to a lesser extent. The characteristic cells were large, with profuse cytoplasm which was angular rather than rounded, and were considered to be reticulum cells.

The tissues of 54 control mice were examined microscopically in the 11½ months period, and a further 12 at 18 months, but no neoplasia of lymph nodes was observed.

Host range.

Unsuccessful attempts were made to recover the virus from guinea-pigs, rabbits, weaned rats (*Rattus norvegicus*), and chick embryos after inoculation with infective material. The animals were inoculated IP, and the embryos were inoculated into the yolk sac, allantoic and amniotic cavities, and onto the chorio-allantoic membrane.

A transmissible agent producing typical splenomegaly was recovered from a pool of liver, spleen and lymph nodes of laboratory rats sacrificed 14 days after IP inoculation when one-day old. The inoculum was an unfiltered suspension of virus in mouse tissue, and it is possible that intracellular virus survived in

TABLE 7.

Details of splenomegaly and lymphadenopathy in mice inoculated when less than 24 hours old.

Material	No. in group	Spleen		Inguinal lymph node		Lumbar lymph node		Mesenteric lymph node	
		$\bar{x}\pm$SD	No. exceeding $\bar{x}+2$SD of normal group	$\bar{x}\pm$SD	No. exceeding $\bar{x}+2$SD of normal group	$\bar{x}\pm$SD	No. exceeding $\bar{x}+2$SD of normal group	$\bar{x}\pm$SD	No. exceeding $\bar{x}+2$SD of normal group
WM1 blood unfiltered	23	138±32*	19	15·7±7·7*	15	14·5±7·7*	16	61·6±23·3*	6
WM1 blood filtered 320 mμ.	31	135±61*	23	17·6±10·6*	13	13·8±10·7*	17	87·8±45·6*	18
Normal blood unfiltered	29	77±11	1	7·3±2·7	1	5·0±2·8	2	47·5±11·8	1

*=Values that are significantly different from those of the controls.

TABLE 8.

Some general properties of the WM1 virus.

Treatment	Survival
Heat (56° C./30 mins.) Control (4° C./30 mins.)	− +
Heat (37° C./24 hours) Control (4° C./24 hours)	− +
Storage (4° C./3 weeks) Storage (−60° C./6½ months)	+ +
Lyophilization	+
50 p.c. ether (4° C./24 hours) Control (50 p.c. saline/4° C./24 hours)	− +
0·5 p.c. formalin (4° C./3 days) Control (4° C./3 days)	− +
5 p.c. ethanol (4° C./24 hours) Control (similar dilution with saline/4° C./24 hours)	+ +

+ = survival; virus recovered on subinoculation.
− = complete inactivation; virus not recovered.

heterografts. Therefore, two further attempts at infection of one-day old rats were made, using infective filtrates from 320 mμ. A.P.D. Gradocol membranes. These groups of rats were killed 14 days later, but the virus was not recovered. No agent was isolated from 12 other uninoculated weaned rats of the same colony.

Filtration.

It was found that the virus was consistently filterable through Gradocol membranes with A.P.D. of 320 mμ. From the results of one experiment shown in Table 7, it can be seen that typical splenomegaly and lymph node enlargement were present in mice examined four months after inoculation with unfiltered infective material and with 320 mμ. filtrate of the infective material, while uninfective control material did not produce such enlargements. Three of 31 mice receiving the filtrate had spleens with indices in the range 225-384, and 3/31 had inguinal or lumbar nodes with indices in the range 40-60. There were no enlargements of this order in the controls. The production of greatly enlarged lymph nodes by filtered preparations confirmed that they were associated with the viral infection.

Effect of various procedures.

Quantitation of the WM1 virus at the present time is relatively insensitive. It was therefore considered to be more practicable to determine whether or not a certain procedure completely inactivated the virus rather than to attempt to obtain inactivation gradients. A certain amount of useful information was

obtained with this method and is listed in Table 8. The virus material used was the supernate of a 10^{-1} saline suspension of infected liver and spleen, clarified at 2,500 r.p.m. for 15 minutes.

DISCUSSION.

It is considered that the WM1 virus described here was isolated from the wild mouse, although it was not possible to confirm this, as material from the mouse had not been preserved. Further evidence that appeared to indicate that such an agent is actually fairly common in wild mice in Queensland, was the isolation of several other apparently similar agents which also produced splenomegaly and lymphadenopathy. At present, means of showing whether these strains are identical are not available, as attempts to demonstrate neutralizing antibodies to the virus, in mice, rats, guinea-pigs or rabbits, have so far been unsuccessful.

It has been clearly shown that this filterable agent, presumably a virus, caused a persistent, chronic infection in experimentally infected mice, with splenomegaly and lymphadenopathy. There was apparently a permanent viraemia, as virus could be recovered from the blood at any time throughout the life of the mouse. Further study of infective blood indicated that virus was present in serum, in approximately the same concentration as in the cell fraction. No particular tissue tropism of the virus was demonstrated, but the studies were limited to spleen, liver and lymph nodes. The host-parasite relationship in this type of infection is interesting material for speculation.

Splenomegaly and enlargement of lymph nodes were the most striking macroscopic characteristics of the infection. The spleens were usually only moderately enlarged, but a small proportion showed more massive enlargement, the significance of which is not yet fully understood. Lymph node enlargement ranged from slight to massive and, in the mice examined, was essentially a medullary hyperplasia involving mainly plasma cells. The hyperplasia may have been a direct effect of local multiplication of the virus in spleen and lymph nodes, or due to some indirect effect, perhaps hormonal. A higher incidence of greatly enlarged lymph nodes was observed in females than in males.

The neoplasms seen in a small proportion of infected mice were of the same type as those seen in the 18-month control mice, but were present at an earlier stage. This low increase in the incidence of neoplasms in infected mice during the first 11½ months was seen both in mice infected as litters and in mice infected at weaning. Although the difference in incidence between infected and control mice was not significant, it was enough to suggest that the virus infection somehow tended to alter the hosts' susceptibility to reticular neoplasms.

Comparison of this virus with others on the basis of behaviour in mice is difficult because of possible variations in virulence of different strains of a virus and the fact that different strains of mice have been used by different workers. However, it appears that this is a virus which has not previously been described,

either in laboratory or wild mice. The evidence so far obtained suggests that the WM1 virus essentially causes disease of the reticular tissue rather than of the central nervous system, lungs or liver. The viruses belonging to these three latter broad groups can probably be excluded as unlikely to be related to the present virus. Two other viruses which would also appear to be distinctly different are the mammary tumour virus, which was isolated from wild mice by Andervont (1952), and polyoma virus. Specific comparison with various other viruses must await development of suitable immunological techniques with the WM1 virus.

An interesting virus causing reticular disease is that of Taylor and MacDowell (1949), which resembled WM1 in that it produced hyperplastic splenomegaly and lymphadenopathy, but differed as there was also a distinct transient leukemia followed by recovery, a development of immunity to homologous challenge and ready transmission by contact. With the WM1 virus, the disease develops too slowly to allow such challenge experiments to be attempted, and no infection of contact mice in the same tin has yet been observed.

Other viruses causing notable reticular diseases in mice are those producing leukemia. The virus isolated by Gross (1957) from AK mice resembled the WM1 virus, in that it produced splenomegaly and enlarged lymph nodes in C3H mice inoculated soon after birth or at weaning, and was heat-labile and sensitive to ether. It differed, however, insofar as it produced a definite increase in the incidence of leukemia. These similarities may be only superficial, but the association of the WM1 virus with prolonged hyperplasia rather than necrosis, the apparent limitation of the hyperplasia to the lymphoid tissue, and the suggestion that some infected mice develop lymphoid neoplasia at an earlier age than the controls, could be interpreted as evidence that this virus perhaps bears some basic resemblance to certain mouse leukemia viruses.

Acknowledgments. Dr. Thelma B. Dunn, National Cancer Institute, Bethesda, Professor E. S. J. King, Pathology Department, University of Melbourne, and Dr. D. Metcalf, Walter and Eliza Hall Institute of Medical Research, Melbourne, kindly provided a diagnosis of certain histological material. Dr. M. D. Innis, Haematologist, Princess Alexandra Hospital, Brisbane, also generously gave considerable time to study and discussion of the histopathology. Thanks are due to Dr. E. H. Derrick and Dr. R. L. Doherty of this Institute for continued interest and advice. Finally, the valuable and patient assistance of Mr. W. Scott and Mr. R. Campbell throughout this work is gratefully acknowledged.

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FURTHER EVIDENCE AGAINST THE GRAFT-VERSUS-HOST HYPOTHESIS IN RENAL TRANSPLANTATION[1, 2]

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(*Accepted for publication 30th October, 1961.*)

SUMMARY.

The infiltrating cellular reaction in rejected renal homotransplants has been the subject of scrutiny in this paper. One widely held view has been that these cells are of graft rather than of host origin. Additional evidence favouring the opposite view that the cells are of host origin is here brought forward to supplement the evidence put forward by the present authors in a previous paper.

In this study the infiltrating cellular response was examined in two experimental situations – following pre-treatment of the host with massive doses of nitrogen mustard and following labelling of the host with tritiated thymidine.

The cellular infiltration was found to be inhibited by supra-lethal doses but not by a lethal dose of nitrogen mustard.

Following labelling of the host with tritiated thymidine a variable percentage of the infiltrating cells in the rejected kidney showed evidence of labelling. This percentage was low in first-set transplants but very high in a second-set transplant, as might be predicted from theoretical considerations. This finding has implications for theoretical immunology beyond the scope of the present enquiry.

INTRODUCTION.

In homotransplantation studies the possibility of a "graft-versus-host" reaction arises when the transplanted tissue itself contains or comprises immunologically competent cells. This "graft-versus-host" concept first had its genesis in observations of the interstitial cellular infiltrate found in rejected renal transplants but may have been founded upon a misinterpretation of these findings. The "graft-versus-host" interpretation of this cellular infiltrate, arrived at independently by different workers (Simonsen, 1953; Dempster, 1953a; Darmady, Dempster and Stranack, 1955), was founded on a considerable body of indirect experimental evidence and received wide currency until very recently, when it was first formally challenged on the basis of the results of irradiation directed to the renal homograft itself (Fowler and West, 1960).

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[1] Read at the Annual General Scientific Meeting of the Royal Australasian College of Surgeons, Adelaide, August, 1960.

[2] Supported in part by a grant from the Anti-Cancer Council of Victoria.

The present studies were undertaken because, as Simonsen (1960) himself pointed out, in discussion of those irradiation findings, the problem is still not completely resolved. This paper presents additional experimental evidence favouring the view that the origin of the infiltrating cells is from the host, and exposes at least one likely source of the contradictory findings which have followed efforts to suppress the immune response of the host.

EXPERIMENTAL.

In considering the acceptable experimental approaches to this problem of determining the host or graft origin of the infiltrating cells, three obvious solutions suggest themselves; firstly, to inhibit the immunological response of the host; secondly, to inhibit any potential immunological capacity which the interstitial cells of the donor kidney might possess; and thirdly, to find some suitable means of labelling the infiltrating cells in such a way that their origin can be determined. Since our findings following irradiation of the donor kidney have already been reported in detail elsewhere, we shall present here only the results of our experiments along the lines of the other two possible approaches.

In brief, these have consisted of, firstly, interference with the immunological response of the host by pre-treatment with lethal or supra-lethal doses of nitrogen mustard, and secondly, auto-radiography of rejected renal homotransplants, utilising tritiated thymidine as a label for the host's immunologically competent cells. This label is rapidly incorporated into the cells of the host and is subsequently unavailable to the cells of the transplant. Apart from stating that this substance is at present the label of choice for the particular purpose in view, it is not proposed to detail its special advantages which can readily be found in various recent publications (Maloney and Patt, 1958; Cronkite *et al.*, 1959; Lajtha and Oliver, 1959).

NITROGEN MUSTARD PRE-TREATMENT OF THE HOST.

Methods:

Five renal homotransplants were carried out between adult mongrel dogs following massive nitrogen mustard administration to the host. In the animal receiving the highest dose of nitrogen mustard (3 mg./kg.) this was administered as equal divided doses of 1·5 mg./kg. on the day of transplantation and also 24 hours earlier, in the hope of minimising acute toxicity from such a large dose of the drug. In addition, three separate daily intramuscular injections of 100 mg. of cortisone acetate were given to this animal on the day of transplantation and on the two preceding days, again in the hope of favouring even short-term survival from such a massive dose of mustard, and perhaps potentiating its action against the host's immune response. The other four animals did not receive cortisone, and the dose of mustard each received is shown in Table 1.

The transplants were performed in each case by direct end-to-end anastomosis of the renal vessels to the femoral vessels of the host, using continuous running sutures of 6-0 arterial silk. The ureter was brought out as an intubated cutaneous ureterostomy, draining urine into a plastic collection bag, as described in previous experiments (Fowler and West,

1960). Antibiotics were administered as single daily intramuscular injections of penicillin, streptomycin and tetracycline.

Histological material was obtained in two experiments (1 and 2) by open wedge biopsy of the transplants on the fourth post-operative day, and in all experiments at the time of graft removal, which was when the transplant had ceased to function (Table 1).

Results:

The transplants functioned and secreted urine as long as the hosts survived, which was usually not more than four days (Table 1). In all five transplants the vascular anastomoses were patent and free of thrombi at the time of graft removal.

TABLE 1.

Effect of massive nitrogen mustard pre-treatment of the host on cellular infiltration of renal homografts.

Experiment No.	Dose—(mg./kg.)	Time of graft removal (p.o.d.)	Time of death (p.o.d.)	Histological appearances
1	1	6th	23rd	Typical cellular infiltrate of homograft rejection
2	3	4th	4th	No leucocytic infiltration
3	2	2nd	2nd	No leucocytic infiltration
4	2	4th	4th	No leucocytic infiltration
5	1·5	4th	4th	No leucocytic infiltration

In four out of five of these renal transplants none of the lymphocytic or plasma cell infiltration typical of a four-day renal homograft (Fig. 1) was found (Fig. 2) but these four did show evidence of capillary congestion and slight interstitial haemorrhage similar to that already described in irradiated autotransplants (Fowler and West, 1960).

In the host receiving the smallest, although lethal, dose of nitrogen mustard (1 mg./kg.) a fairly typical homograft reaction was observed, with characteristic lymphocytic and plasma cell infiltration, to which were added the features of vascular congestion and interstitial haemorrhage already described in the other transplants.

Autopsy examination of the bone marrow, lymph nodes and spleen of the host animals was made in three out of four experiments (Nos. 3, 4 and 5) in which the homografted kidneys showed no cellular infiltrate. In these animals, as might be expected, severe hypoplasia of the bone marrow, lymphoid and splenic tissues was found. Haemorrhagic pneumonia, and necrosis of gastro-intestinal epithelium were associated features which almost certainly were contributory causes of death.

AUTORADIOGRAPHIC STUDIES WITH TRITIATED THYMIDINE.

Methods:

Three experiments were performed, comprising two first-set renal homotransplants in mongrel dogs, and one second-set renal transplant in a rabbit (following a first-set skin graft from the kidney donor). The host in each experiment was labelled by a single intravenous injection of tritiated thymidine[3] and material collected for histological study according to the individual protocols detailed below. The specific activity of the tritiated thymidine injected was 360 mc/mM. The two dogs each received a dose of at least 100 μc/kg. This dose provides a very adequate degree of labelling of the whole animal as shown by Maloney and Patt (1958) in their detailed studies of daily marrow and peripheral blood smears in dogs. A lower scale of dosage, 55 μc/kg, also provided, as events turned out, an adequate degree of labelling of the host in the single rabbit experiment.

Daily smears of the host's peripheral blood were made and biopsies of bone marrow and the transplanted kidneys were performed on the days indicated. In all experiments biopsies were taken from the donor's contralateral kidney at the time of transplantation, and from the spleen and one kidney of the host at the time of removal of the rejected transplants. These normal donor and host kidney sections provided useful control material for comparison with autoradiographs from the transplants, and received the same processing. The autoradiographs were prepared according to standard techniques (Joftes, 1959) employing Kodak NTB-2 emulsion.[4] Staining was carried out through the emulsion, using haematoxylin and eosin for tissue sections and Giemsa stain for the blood and bone marrow smears.

The renal homotransplants in the dogs were performed by the same technique as that employed in the nitrogen mustard experiments, anastomosing the renal vessels to the femoral vessels of the host. In this situation the transplants were readily accessible for serial biopsies simply by opening and resuturing the thigh wounds.

The technique of performance of the renal transplant in the rabbit was essentially that of Nathan *et al.* (1961), utilising direct end-to-end anastomosis of the donor aorta and vena cava to the carotid artery and jugular vein of the host by means of continuous 7-0 silk sutures, the open ends of aorta and vena cava then being ligated. The ureter was brought out as an intubated cutaneous ureterostomy without any form of collecting apparatus, allowing urine to drip freely from the open end of the tube.

Experiment 1. The host, weighing 12·7 kg., was injected with 1·5 mc. of tritiated thymidine 18 hours prior to the performance of a first-set renal homotransplant. Biopsies were taken from the transplant on the first and fourth post-operative days and on the fifth post-operative day the transplant was removed when it ceased to function. Daily smears were taken from the bone marrow by needle aspiration biopsy.

Experiment 2. 1·3 mc. of tritiated thymidine were injected intravenously into the host dog, weighing 13 kg., four hours prior to renal transplantation. The transplant was biopsied on the first and fourth post-operative days and was removed when it ceased to function on the sixth post-operative day. Bone marrow biopsies were performed on the first, third and fourth post-operative days.

Experiment 3. Seven days prior to the renal transplantation in the rabbit a full thickness graft of auricular skin from the prospective kidney donor was sutured to a matching full thickness defect on the host's left ear, and was observed daily without dressings. This graft became vascularised on the fourth post-operative day and on the sixth post-operative day showed the superficial ecchymoses characteristic of graft rejection. On the seventh post-

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[3] Schwarz Bio-Research Incorporated, Mt. Vernon, New York.

[4] Kodak Special Sensitised Products Division, Rochester 4, New York.

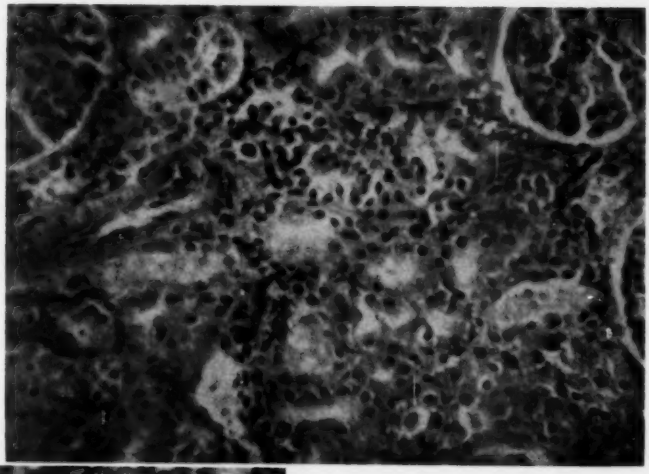

Fig. 1. Experiment No. 5. Four-day renal homograft following pre-treatment of host with 1·5 mg./kg. of nitrogen mustard. Absence of glomerular and perivascular infiltrate. (x 100.)

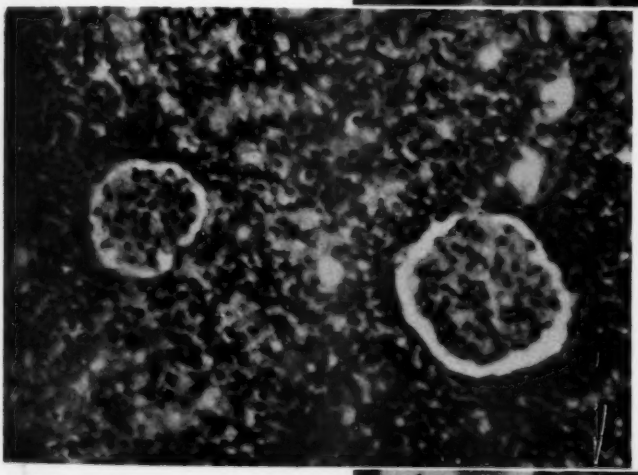

Fig. 2. Typical cellular infiltrate of homograft reaction on 4th post-operative day (first-set dog kidney homotransplant from tritiated thymidine experiment No. 1). (x 100.)

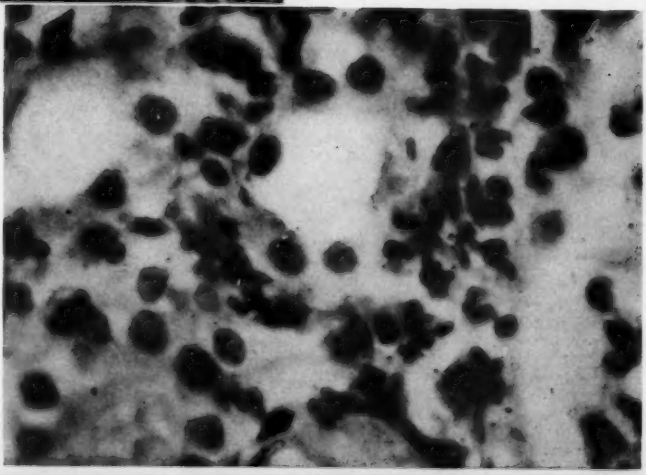

Fig. 3. Autoradiograph of four-day first-set renal homograft in dog labelled with 100 microcuries/kg. of tritiated thymidine (Experiment No. 1). Cells among the interstitial infiltrate showing grain counts of three or more grains per nuclear area are labelled cells. (x 430.)

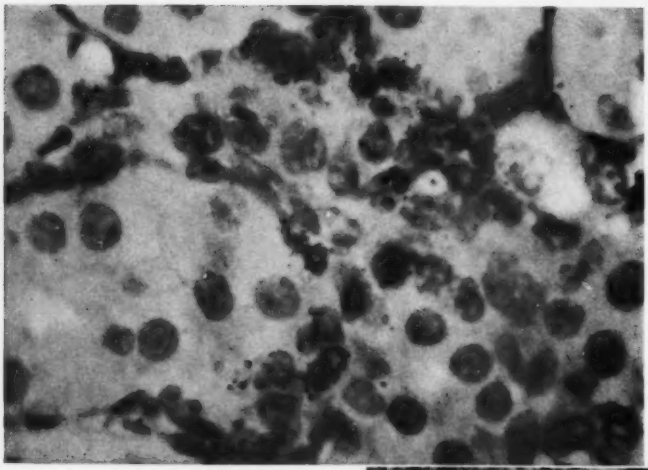

Fig. 4. Autoradiograph of interstitial cellular infiltrate in 18-hour second-set renal transplant in rabbit (Experiment No. 3). (x 430.)

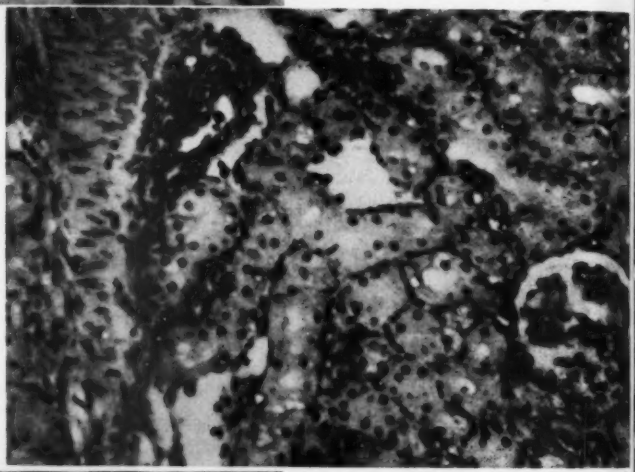

Fig. 5. Early perivascular and interstitial cellular infiltrate 18 hours after second-set renal homotransplantation in rabbit (Experiment No. 3). (x 100.)

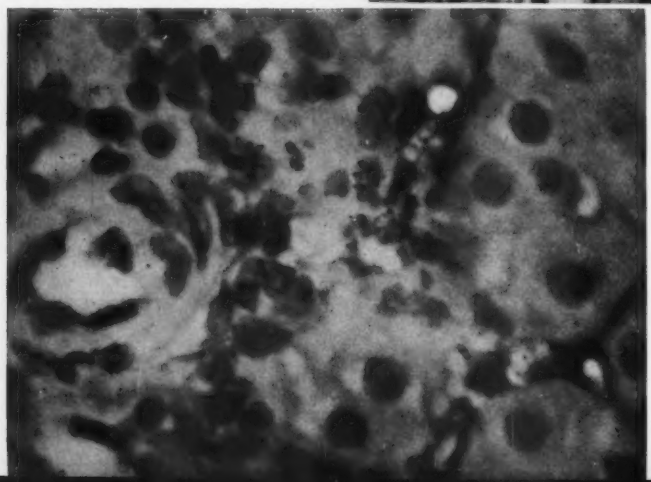

Fig. 6. Autoradiograph of early interstitial cellular infiltrate and small focus of perivascular cellular proliferation in Experiment No. 3. Higher incidence of labelled cells between tubules than in focal cellular proliferation. (x 430.)

operative day the rejected skin graft was removed and the host, weighing 4·5 kg. was injected with 250 μc. of tritiated thymidine four hours prior to the performance of a second-set kidney transplant.

Eighteen hours after renal transplantation the wound was re-opened to expose the transplant, which was well vascularised and bled profusely when the upper pole was excised for biopsy. A steady flow of urine from the transplant was still evident the following day, but on the third post-operative day urine flow had ceased and the transplant was removed. The vascular anastomoses were still patent but the kidney was markedly enlarged, congested and haemorrhagic – appearances typical of a second-set renal homograft reaction (Egdahl and Hume, 1956).

Results:

The peripheral blood and bone marrow smears, and the spleen sections, apart from providing confirmatory evidence of adequate labelling of the host, were otherwise non-contributory.

In the host kidneys, removed at the time of transplant rejection, only a very occasional labelled cell was seen, either in glomerular capillary tufts or in the lumen of small blood vessels. These sections, and those from the unlabelled donor kidneys, provided the control material for assessing background grain count and artefactual appearances resulting in tissue sections prepared by the above techniques. No difficulty was encountered in recognising labelled cells when these were present in any kidney section, which in the control donor kidneys was not at all and in the host kidneys was extremely seldom.

In the sections of the transplanted kidneys, in the first-set experiments, labelled cells were a relative rarity in the earliest biopsy material taken on the first post-operative day. By four days, however, there was a definite emergence of infiltrating cells which bore unmistakable evidence of labelling (Fig. 3). The percentage of cells showing the label was very variable among the different foci of infiltrating cells throughout the section. In experiment 1 it was estimated that, at a maximum, not more than 10-15 p.c. of infiltrating cells bore the label. In experiment 2 the percentage of labelled cells was not even as high as this, barely reaching 5 p.c. even in the areas displaying labelling.

By contrast, however, in the second-set experiment a very high percentage of labelled cells was present in the infiltrate in the 18-hour specimen (Fig. 4), even though the dose of labelled thymidine administered to the host was relatively less than in the two first-set experiments. Although at this time the extent of cellular infiltration was appreciably less than in the three to four-day first-set transplants, early periglomerular and perivascular foci were already present (Fig. 5), together with some interstitial infiltrate between tubules. In some areas, particularly in between the proximal convoluted tubules, virtually 100 p.c. of these infiltrating cells showed heavy labelling (Fig. 4), whereas in other areas, usually where foci of cellular proliferation were to be found, and where dilution of label was likely, the percentage of labelled cells was lower – perhaps 30-50 p.c. – and the intensity of labelling of individual cells appeared less

(Fig. 6). However, everywhere the incidence and intensity of labelling among the infiltrating cells was strikingly more apparent than in the first-set experiments.

In the three-day sections from this second-set kidney there was a patchy infarction and an excess of pyknotic nuclear debris, making the identification of labelled cells uncertain. The impression gained was that there had been a diluting out of label from the cellular infiltrate, presumably by multiplication and division of the cells concerned, but this later material contributed little of value.

DISCUSSION.

Evidence from many sources now supports the validity of the graft-versus-host concept in relation to transplants of immunologically competent cells (Simonsen, 1957; Trentin, 1957), but the prime concern of this present study is whether the interstitial cells in a transplanted kidney are immunologically competent cells capable of reacting against the foreign proteins of the host. Unlikely as it may perhaps appear, this possibility cannot be ignored, since Billingham and Silvers (1959) have shown that even skin homografts are capable in the chick embryo of eliciting reactions which appear to be graft-versus-host in type. Presumably the connective tissue elements of these skin grafts contain interstitial cells of a reticulo-endothelial character which can manifest immunological competence against foreign cells.

The possibility of graft-versus-host reactions in renal homografts has become a question of major importance to the future practical application of renal transplantation. It is essential to know whether efforts need be directed solely towards rendering the host tolerant to the kidney, or whether, as has been suggested (British Medical Journal, 1958; Mitchell, 1959; and Küss *et al.*, 1960) the donor kidney must likewise be rendered tolerant towards the host. The results of the two different experimental approaches employed here to answer this question will now be separately discussed along with other evidence.

(a) *Effects of damaging the host's immune response.*

It has been apparent for some time that neither total body irradiation (Dempster, 1953a; Baker and Gordon, 1955) nor radio-mimetic agents (Baker *et al.*, 1952) in sub-lethal dosage have any appreciable effect upon the pattern of rejection of renal homografts, facts which lend credence to the graft-versus-host interpretation. Even considering the effects of lethal doses of such agents, however, one could predict that, even though the host animals might die, the population of immunologically competent cells within such hosts would not be wholly destroyed. It is not surprising, therefore, to find in one of our own experiments that although a dose of 1 mg./kg. of nitrogen mustard was sufficient to kill the host, it did not appear to suppress the homograft reaction, whereas in four other animals the administration of supra-lethal doses of this agent produced a striking suppression of the cellular infiltrating response.

Following the initial failure of mustard alone in our first experiment, a combined onslaught on the host's immune response was made in the second experiment, using pre-treatment with nitrogen mustard and cortisone. This pre-treatment with cortisone does not really obscure the issue, since cortisone alone has been unsuccessful in preventing the rejection of renal homografts (Persky and Jacob, 1951; Dempster, 1953b), and the results of the remaining experiments confirm that nitrogen mustard alone can achieve a comparable effect if given in high enough dosage. In the previous experiments of Baker *et al.* (1952) not only was nitrogen mustard given in sub-lethal dosage, but it was also administered in fractionated doses during the post-operative period, so that no discrimination between the effect on the host and any possible effect on the graft could be made.

We conclude from our present observations that agents capable of damaging the host's immunologically competent cells are capable, when administered in high enough dosage, of inhibiting the host's contribution to the homograft rejection pattern. In support of this contention is the more recent finding by Hume *et al.* (1960) that the cellular infiltrating response can regularly be suppressed by whole body irradiation of the host, but only in the supralethal dose range.

(b) *Results of labelling the host's immunologically competent cells.*

The autoradiographic studies by means of tritiated thymidine labelling of the host's immunologically competent cells are of considerable interest in showing that a proportion at least of the cellular infiltrate in rejected renal homografts is undeniably of host origin. However, we find the results of our first-set experiments far less compelling than the results of the nitrogen mustard experiments as evidence against the graft origin of some of the infiltrating cells. Less compelling also are the results of Porter and Calne (1960). Working with tritiated thymidine, in first-set experiments only, they found a relatively low incidence of labelled cells among the infiltrate, comparable with that reported here. We feel, nevertheless, that there is now sufficient evidence overall to justify abandoning the hypothesis of the graft origin of these cells. The results of our second-set experiment were particularly compelling in this regard.

In the first-set transplants, however, the failure to find more than a small proportion of infiltrating cells carrying the thymidine label is not necessarily evidence against the host origin of the remaining unlabelled cells, as might be foretold from some brief flights into theoretical immunology. If a large majority of the immunologically competent cells labelled in the host are capable of responding to the particular transplant antigen, then one would expect to find a high percentage of such cells infiltrating the transplant. (This would be in keeping with the "instructive theory" of the active rôle of antigen in inducing immunity.) However, if only a very small fraction of these cells in the host is capable of responding to the specific antigen by segregation as a clone (in keeping with the "elective" or "clonal selection" theory (Burnet, 1959) of the

passive rôle of antigen), then one might expect a very low percentage of labelled cells to be found in the cellular infiltration in a first-set graft. Such we found to be the case in our first-set experiments. If, on the other hand, the host could be labelled at a time of stimulation and active proliferation by such a clone, then a very high percentage of labelled cells might be expected in a second-set graft, and such we found to be the case.

The results of our second-set experiment, therefore, begin to take on a new and more significant interest than that for which they were first intended. Not only do they afford very cogent supporting evidence in favour of the host origin of the infiltrating cells, but they reveal one facet of the cellular kinetics of the immune response which would seem to have a direct bearing on current immunological theories, particularly the clonal selection hypothesis of the nature of the immune response.

It would seem that further work with tritiated thymidine as a label in transplantation studies should prove to be a most useful tool in the future elucidation of the cellular kinetics of the homograft reaction.

(c) *Other evidence.*

A few instances of unusually prolonged renal homograft survival without cellular infiltration have been reported in the dog (Mannick *et al.*, 1959) and in man (Dealy *et al.*, 1960) following total body irradiation and bone marrow transplantation to the host. However, the presence of donor cells in the host's circulation modifies the course of renal (Egdahl and Hume, 1957) or skin (Murray *et al.*, 1959) homograft rejection by means as yet unknown, so that it is desirable to define the answer to our problem in situations lacking this complicating factor.

The results of a totally different type of experiment (Wheeler and Corson, 1960), employing genetic labels for both host and grafted tissues, support the same general thesis advanced in this paper but are based on implants of kidney slices rather than true vascularised renal transplants, so that the results are not fully comparable.

Complementary to our earlier finding that massive single dose irradiation directed to the transplant failed to prevent the typical cellular infiltration is a very recent study by Banks *et al.* (1961) showing that daily intermittent irradiation of renal transplants is likewise ineffective in modifying rejection patterns.

CONCLUSIONS.

Taking together the results of this present study, our earlier findings, and all the other published evidence, we conclude that there is no longer any good evidence for believing that the infiltrating cells are of graft origin. There is abundant evidence to show that they come from the host. The important implication for the future clinical application of renal homografts is that efforts should be directed to modification of the host's immune response rather than to attempts to modify a hypothetical response by the graft.

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METHODS IN IMMUNOCHEMISTRY OF VIRUSES

I. EQUILIBRIUM FILTRATION

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(*Accepted for publication 31st October, 1961.*)

SUMMARY.

An apparatus for the separation of free antibody from the virus-antibody complex is designed and tested.

It is demonstrated on the influenza virus-antibody system that the technique is not biassed by systematic errors and is equally suited to kinetic and thermodynamic measurements.

The sensitivity and reproducibility of equilibrium filtrations are evaluated statistically.

INTRODUCTION.

Although the interaction of antigen and antibody has been studied over the past half century by perhaps the most rigorous and ingenious quantitative methods to be applied to biological systems, immunochemistry has not yet ventured outside its circle of *in vitro* tests, and its preferred antigens, too, can hardly be regarded as representative of the host of antigens against which immune responses in nature are directed. This deliberate narrowing of scope is not altogether surprising: the problems to be faced are complex enough without having to cope with the extra variables and vagaries of *in vivo* tests. Yet, with its powerful techniques immunochemistry could contribute greatly to the exploration of infective antigens and it seems somewhat archaic to make do in practical immunology with methods of assay and evaluation long since superseded in the study of the more readily definable, simpler systems.

The formation of an antigen-antibody complex gives rise to a new species of solute and simultaneously reduces the concentration of the initial reactants. Thus, in principle, any method capable of either separating the components of a heterogeneous system, or of estimating any one of the species making up the system, is suitable for determining the parameters of neutralization. In practice, the handling of viruses and their antibodies presents some special difficulties. Preparations of these antigens are usually impure and of low concentration, with little if any information about the antigenic groups, and antisera are seldom available in quantity. Thus if such tests are to be of any use, they must not only be simple to perform but also economical and exceptionally sensitive. The

experimental approach here is essentially eclectic: from among an array of potential techniques such combinations are to be selected as will – with the reagents at hand – quantitatively answer particular questions. We start with some methods based on the size of the reactants.

The classical procedure of equilibrium dialysis employs semipermeable membranes that retain macromolecules and let solvent and crystalloids pass freely. In immunochemical terms this restricts the method to work on artificial antigens, that is, to haptenic groups coupled to large carriers. The equilibrium is established between the separate small hapten and antibody in one compartment, and the concentration of free hapten is then determined by sampling the other compartment where antibody cannot pass. The system, of course, is also suited to competitive tests: the complete antigen, added to the antibody compartment, will shift the distribution of hapten and thus, indirectly, the binding between antigen and antibody can be studied. If the haptenic groups are not available in isolated form or, as is the rule with natural antigens, not even known, the method breaks down as it cannot sift reactants of comparable size.

With large particulate antigens such as cells, microorganisms and viruses, however, the technique is once more feasible. All that is needed is a membrane of suitable pore size to allow the passage of antibody molecules and retain the large antigenic particles. Such membranes, owing largely to the work of Zsigmondy and his school, are now readily available in standardized form and provide a simple technique for the separation of antigen and antibody.

Since the diffusion coefficient of antibody molecules would be about ten times smaller than that of the average hapten, establishment of osmotic equilibrium in a virus-antibody system would take much time, allowing denaturation or inactivation of the reagents. We opted therefore for ultrafiltration as a means of separating free antibody from the virus-antibody complex.

MATERIALS AND METHODS.

Diluent. The Ca-Ma-saline of Fazekas de St.Groth, Graham and Jack (1958) was used throughout.

Virus. Strain 15 of swine influenza virus (Shope, 1931) was grown allantoically in 11-day chick embryos. After two days' incubation at 35° C. the allantoic fluids were harvested, absorbed with 2 p.c. human erythrocytes, and the virus eluted into one-tenth of the original volume. Such concentrates were preserved by 0·08 p.c. sodium azide, and stored at 4° C.

Titration of virus. Haemagglutinin tests were done in plastic trays, as described by Fazekas de St.Groth and Graham (1954), but using 5 p.c. fowl red cells instead of 10 p.c. Infectivity was assayed in surviving bits of allantois-on-shell, by the method of Fazekas de St.Groth and White (1958).

Antiserum. A rabbit was given 5000 agglutinating doses of virus intravenously on five consecutive days, and then again on day 40, 60 and 80. The serum obtained from the 83-day bleed was stored at – 15° C., and used without inactivation.

Titration of antibody. Antihaemagglutinin tests were performed according to Fazekas de St.Groth, Withell and Lafferty (1958) against four agglutinating doses of SW virus.

EXPERIMENTAL.

Instrumentation.

Apparatus. The choice of apparatus was governed by the need of handling small volumes (of the order of 1 ml.) and a large number of samples at a time. Of the commercially available filtering devices the Swinny-apparatus comes closest to meeting these demands. It has, however, certain disadvantages: passage through the narrow opening of a syringe makes it almost impossible to avoid occasional air-locks between filter and sample; some of the filtrate is retained in the cavity and duct of the instrument after passing through the filter; and the use of a syringe makes individual handling necessary. To correct these shortcomings the apparatus shown in Fig. 1 was designed.

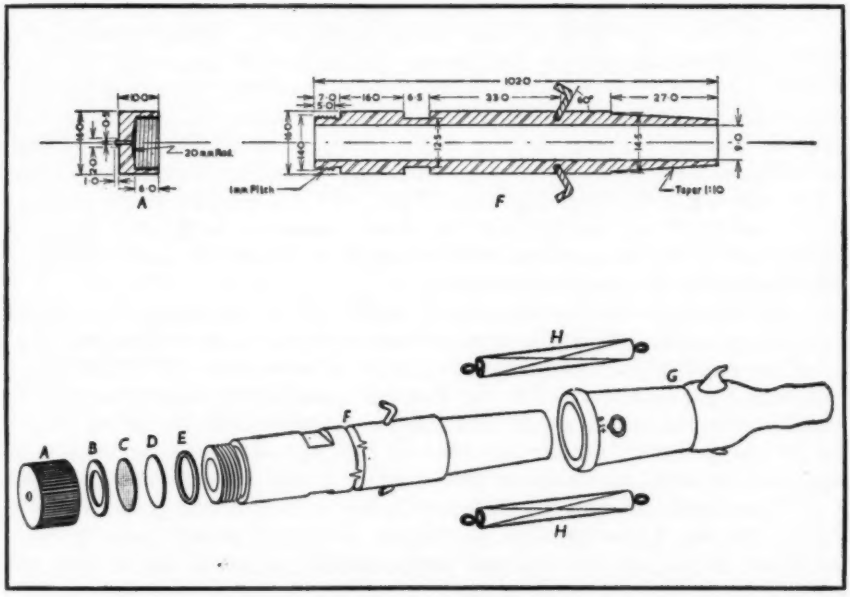

Fig. 1. The filter assembly.

A: Lower part of stainless steel filter housing.
B: Lower, flat teflon gasket.
C: Perforated backing plate.
D: Filter membrane.
E: Upper, ring gasket.
F: Upper part of filter housing.
G: Quickfit B14 fitting, connected to compressed air source.
H: Springs to hold F and G together.

All measurements are in millimetres.

The filter membrane (D), of 13 mm. diameter and 1 cm.^{2} effective area, rests on a stainless steel crib-plate (C). Teflon gaskets (B and E) seal it on top and bottom. The housing is of stainless steel tubing, with a capacity of about 6 ml. above the filter membrane, and a dead volume of about 0·05 ml. between filter and lower aperture. The top end of the upper tube (F) is conically turned, to take interchangeable glass fittings (Quickfit B14). These glass links (G) are connected through a manifold to a compressed air source.

Since the upper tube has no narrowing, air-locks cannot spoil the experiment; the volume of fluid retained after filtration is equivalent to a single drop, and usually less than 5 p.c. of the sample; and any number of tests can be run in parallel, the connection of each tube taking only a few seconds.

Filters. The criteria for appropriate filters are (1) high rate of flow; (2) controlled pore size, to allow passing of antibody while quantitatively retaining virus; (3) inertness towards all reagents; and (4) minimal retention of fluid.

(1) Of the two kinds of filter[1] judged suitable on general grounds, the Millipore filters had, over the pore size range of 10 to 100 mμ, about ten times higher flow rates than the corresponding Membrane filters. Using either type, it was found that with protein solutions such as serum, and especially with impure virus preparations, the rate of flow dropped considerably after as little as 0·5 ml. of a 1 p.c. protein solution had passed. Clogging of membranes could be prevented readily by adding about 5 p.c. (w/v) Kieselguhr to the preparation to be filtered, and letting it settle for about a minute in the filtering apparatus before turning on the pressure. This amounts to inserting a coarse clarifying filter in front of the ultrafilter membrane.

(2) When testing for permeability, both kinds of membrane were found satisfactory. Although Membrane filters have a wider scatter of pore size than Millipore membranes, the appropriate grades (Membranfilter 10, Ultrafeinfilter "g", and Millipore VM and VF) all have the rated characteristics required to separate influenza viruses ($\sim$ 80 mμ diameter) from antibody ($\sim$ 24 mμ longest axis). No virus could be detected either as haemagglutinin or as infective units when a preparation of virus was filtered under 1 atm. pressure through one of the above listed membranes. This sets an upper limit to the permeability at 10^{-5}. The next higher grade of membranes, of 100 mμ average pore diameter, let about 10 p.c. of the virus pass under similar conditions and is thus not suited to work on the influenza group.

(3) When similar experiments were performed to find out whether the membranes that retained virus would pass antibody, no quantitative recovery could be obtained, irrespective of pore size. Obviously, both kinds of membrane interacted with γ-globulin. To overcome such a complication, we first tried increasing the ionic strength of the medium to be filtered, and shifted the

[1] Gelatine "Membrane filters" (Membranfiltergesellschaft, Göttingen) and cellulose acetate "Millipore filters" (Millipore Filter Corporation, Bedford, Mass.).

pH to either side of neutrality. Although some of these changes as well as their combination improved recovery, the final yields still remained below the input. We then attempted to saturate the binding sites of the membrane with a protein homologous, at least in this respect, to antibody. A solution of gelatine was found satisfactory for the purpose. The summary of experimental results is given in Table 1.

TABLE 1.

The binding of antibody by filter membranes.

Diluent		Membrane	Filtrate	
molarity	pH		log titre	per cent. recovery
0·15	7·2	untreated	2·20	31
0·15	9·5	untreated	2·23	33
0·15	4·5	untreated	2·11	25
1·71	7·2	untreated	2·53	66
1·71	9·5	untreated	2·47	58
1·71	4·5	untreated	2·50	62
0·15	7·2	gelatine-treated	2·71	100
0·15	7·2	unfiltered control	2·71	100

Anti-SW rabbit serum diluted 1:40 was passed through Millipore VM membranes.

In practice, the saturation of protein-binding sites can be done by passing a 0·5 p.c. solution of gelatine through the membranes in the filter assembly directly before an experiment. This technique, however, leaves the filters with a residual volume of fluid since pressures applicable under ordinary conditions are insufficient to overcome the capillary pressure of fluid in the membranes, and thus cannot clear the lower passage by blowing air through it. An alternative technique proved preferable. The membranes were floated[2] on a solution of gelatine – we used the Standard Medium of Fazekas de St.Groth and White (1958), which contains 0·2 p.c. gelatine in a balanced salt solution – and then dried under an infrared lamp. The dry, gelatine-coated membranes are ready for use, or can be stored at room temperature for any length of time. For all experiments detailed below, the filters were treated with gelatine in this fashion.

(4) The volume of fluid held within the membrane becomes a serious complication only if, to achieve greater accuracy, not only the filtrate but also the retained fraction is tested for the presence of a particular component, and the true ratio of the two is estimated from the observed ratio. This would usually be the case with radioactively labelled reagents, and could lead to large sys-

[2] It is essential that the membranes should come into contact with the fluid on one side only. Dipping will inevitably lock air in at least some of the capillaries, and thus not only defeat the purpose of the treatment but also reduce the effective filtering area.

tematic errors, especially in the regions far from equipartition. The volume of fluid retained was determined gravimetrically. As a check we also passed I^{131}-labelled proteins and KI^{131} through the filters. The latter would essentially measure whether there is an excess of I^- ion in the volume of fluid retained, while the former could also show whether protein was held back preferentially.

TABLE 2.

Capillary volume of filter membranes, and tests for preferential retention of solutes.

Membrane	Capillary volume (μl)	Percentage of solute retained*	
		KI^{131}-activity	I^{131}-globulin activity
'Membranfilter 10'	6·89	0·45	9·60
'Millipore VM'	10·83	0·00	11·02
'Millipore VM' treated with gelatine	9·55	1·15	2·30

* calculated after correcting for capillary volume.

From the results in Table 2 it is clear that both kinds of filter retain measurable volumes of fluid, Millipore membranes about half as much again as Membrane filters (10 μl and 7 μl, respectively). Judged by the KI^{131} activity, the volume would be about 20 μl and 5 μl, respectively. The protein activity gives values of 38 μl and 102 μl, thus indicating that in appropriately treated membranes retention is appreciable though not excessive.

As long as concentrations are compared, the volume of fluid held by the membranes is probably of no importance since it seems to have a composition close to the filtrate. As soon, however, as quantities are measured and referred to the input volume, corrections must be taken even if retention is proportional. In particular, appropriate controls will be needed when the membrane itself is to be tested, say, as a source of radioactivity coming presumably from components above the filter membrane. It is easy to conceive of situations where the contribution of the membrane itself is not only comparable to the sample to be assayed but, indeed, overwhelming.

On the grounds of the combined evidence of this section, gelatine-treated Millipore VM membranes (13 mm. diameter, 50 mμ average pore size) were used in all experiments for the separation of influenza viruses and their antibodies.

The standard test.

Temperature control. During the developmental stage we did not observe special precautions, the tests being run at room temperature (18-20° C.). Virus and antiserum, stored at 4° C. and – 15° C. respectively, were diluted about an hour before the tests in saline kept at room temperature. With dilutions of tenfold or higher, the temperature of reagents could not have been more than a

degree below that of the environment. Since the technique was being designed with thermodynamic measurements in view, experiments were later conducted in constant temperature rooms, set at 4, 22 and 35° C., with a variation of less than 0·5° C. Both the filtering assembly and all reagents were kept at the appropriate temperature overnight.

Preincubation. Equal volumes of appropriately diluted serum and virus were brought together in small test tubes, well shaken and incubated for half-an-hour. As will be seen below (Fig. 3), this time is not critical with reagents of the usual concentrations, since the stage reached after five minutes is indistinguishable from equilibrium conditions. When working with very dilute systems, however, it is essential to perform a preliminary test of the kind shown in Fig. 3. As the rate of collisions between virus and antibody falls away with the inverse product of their concentrations, the period of 30 minutes, greatly in excess under ordinary conditions, might not be sufficient for attaining equilibrium when reactants are present in low concentrations only.

Volumes. Depending on the type of assay used for testing the filtrate, volumes ranging from 0·5 to 5 ml. were pipetted into the upper compartment of the filtering device. Kieselguhr (5 p.c. w/v) was added only exceptionally, when the concentration of total solids approached 1 p.c. and the volume exceeded 2 ml.

Collection of ultrafiltrates. Once in contact with the filter membrane, the samples will start seeping through slowly, even under their own weight. When the glass caps are fitted and the compressed air turned on to give an atmosphere excess pressure, the flow rate rises to about 0·7 ml./min. over the cm.^{2} exposed area of membranes with 50 mμ average pore diameter. Thus, using 1 ml. volumes, the filtration is complete within a minute and a half, and even with the largest volumes it takes less than ten.

Demonstration of unbiassed sampling.

It may be suspected that a period of even a few minutes is sufficient to allow significant drift from the original equilibrium. Intuitively, one might envisage an interplay of two opposed factors. First, the removal of free antibody from the upper compartment would tend to be compensated by dissociation of formed virus-antibody complexes. Secondly, the progressive reduction of volume would favour association. That these two processes exactly balance each other can be shown formally by writing down the two equilibria, the first for the original volume v, and the second for a smaller volume w, after $(v-w)/v$ fraction of free antibody has passed through the filter.

$$\frac{(sV-x)(A-x)}{xv} = K = \frac{(sV-y)\{A-[(A-x)(v-w)/v]-y\}}{yw}$$

where V is the number of virus particles in the system, each with s antigenic sites; A is the number of antibody molecules, x and y the number of antigen-

antibody complexes formed when the volumes are v and w respectively. K stands for the equilibrium constant. It is evident that the condition of equality, that is the constancy of K, is satisfied if, and only if, $x = y$.

It is implied in this treatment that the filter does not discriminate between solvent and the solute of importance, in this case antibody. If filter-solvent, filter-solute and solvent-solute interactions are not negligible, all measurements on the filtrate will be biassed. Evaluation will then have to rely on the more complex model based on the thermodynamics of open systems (Kedem and Katchalsky, 1958).

To test for the presence and degree of such bias, we performed the following experiment. Two doses of SW virus (730 and 365 haemagglutinating units respectively) were taken up in 0·50 ml. saline and mixed with an equal volume of rabbit anti-SW serum diluted 1/10. To allow more extensive testing, the globulin fraction of the serum was labelled with I^{131}. After preincubation at

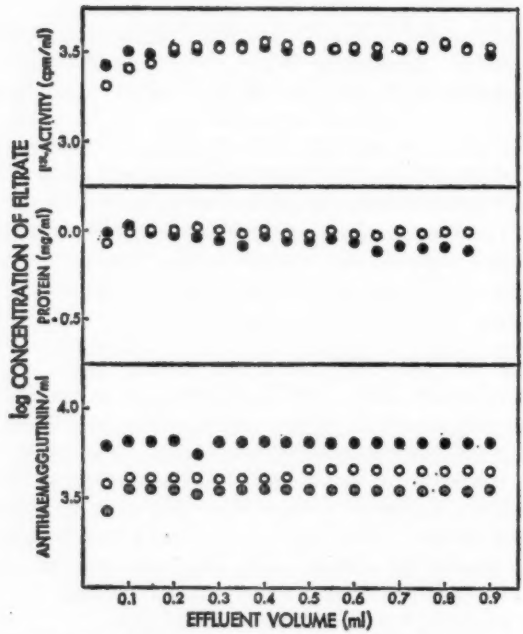

Fig. 2. Variation of the effluent during filtration. A hyperimmune rabbit serum was mixed with 365 (circles) or 730 (dotted circles) haemagglutinating doses of the homologous SW virus, or with saline (dots) as control. After 30 min. incubation the mixtures were filtered through a Millipore VM membrane, and the effluent collected dropwise.

room temperature for 30 minutes, the mixture was passed through a 50 mμ Millipore membrane pretreated with gelatine. The filtrate was collected dropwise, i.e. in 0·05 ml. volumes. Each of the fractions was analysed for protein by the method of Lowry *et al.* (1951), for antibody by the antihaemagglutinin test, and for specific activity in a β-counter.

The curves in Fig. 2 show that, apart from the first two drops of the filtrate, the concentrations vary only within the expected accuracy of the respective method of assay. The first 0·1 ml. has a concentration about 12 p.c. lower than the rest, and its inclusion in the average will lead to an error of about 2 p.c. With larger volumes the error would be proportionately less. For more accurate work, therefore, the first fractions should be considered only as a means of estimating correction coefficients to be applied to the subsequent stationary state, where the concentrations of each solute are stable, i.e. the "exchange flow" has become negligible. We have not used this refinement as the maximal error so introduced is well below the discriminatory power of the usual antibody assay.

Demonstration of equilibrium.

Rate of reaction. Dilutions of virus and antibody were mixed in bulk, and 1·0 ml. lots placed directly into the upper compartment of the filtering device. At intervals up to two hours a set of samples was passed through the filter, and

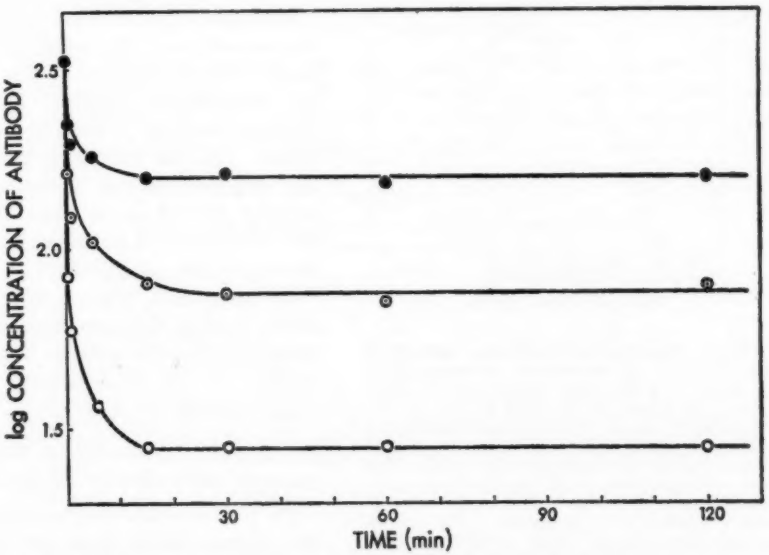

Fig. 3. Rate of virus-antibody union. Anti-SW serum diluted 1:10, was mixed with an equal volume of SW virus diluted 1:5 (circles), 1:10 (dotted circles) or 1:20 (dots), and aliquots filtered at times shown on the abscissa. The ordinate gives the antihaemagglutinin content of the filtrates.

all filtrates assayed for antihaemagglutinin at the end of the test. Fig. 3 gives the result of one such experiment with SW virus and rabbit anti-SW serum.

The initial rate of reaction is rapid and, at the concentrations used, the process practically complete within five minutes. This is reassuring as far as equilibrium measurements go, but precludes accurate measurement of rate constants. The kinetics of association could be studied only on much more dilute systems, indeed, in a range which may well lie beyond the limits attainable by the most sensitive assays for antibody.

Approach from two sides. The most cogent demonstration of equilibrium is to show that the same final relationship of concentrations is reached whether approached from an excess of one or the other component of the system. In two experiments, therefore, the ratio of virus to antibody was gradually changed over a hundredfold range. In the first experiment small doses of antibody were added to the full dose of virus, and samples filtered ten minutes after each addition. The second experiment was set up the other way round: the concentration of virus was gradually increased in face of a fixed volume of antibody.

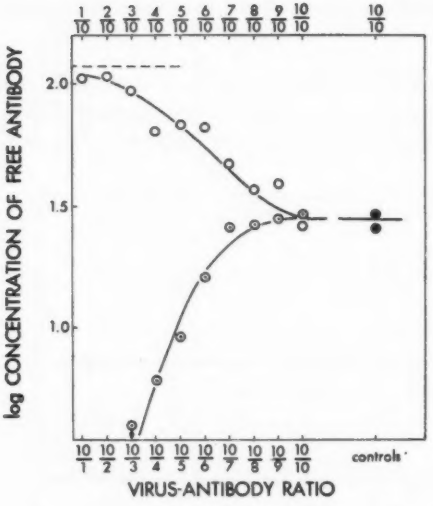

Fig. 4. Approach to equilibrium from either side. To 1·00 ml. volumes of one reagent 0·10 ml. volumes of the other reagent were added every 10 minutes. At the time of each successive addition one of the mixtures was filtered. Antihaemagglutinin titres of filtrates starting at antibody excess are shown by open circles, and their virus-antibody ratio on the top scale. Dotted circles and the bottom scale refer to filtrates starting at virus excess. The control mixtures consisted of equal volumes of virus and antibody, and were filtered after 30 min. incubation. The original antibody titre (a 1:5 dilution of anti-SW serum) is shown by the horizontal broken line; the preparation of SW virus was also used at a dilution of 1:5.

It should be noted that the final concentration of the reagents was about twice as high as in the kinetic experiment described in the previous section. Thus the ten-minute periods following successive additions of one or the other reagent should have been more than sufficient for attaining appropriate interim equilibria.

As seen from Fig. 4, the two curves tend to the same asymptote. Formally this amounts to the definition of an equilibrium, and experimentally to its proof.

Irrelevance of heterologous antigens and antibodies in the system.

To test whether components of the system other than the viral antigen and its homologous antibody contribute to the results, two sets of control experiments were

performed. In the first of these a dose of SW virus (sufficient to bind 80 p.c. of the dose of antibody used) was brought together with homologous antibody, both in the absence and in the presence of different animal sera, either normal or hyperimmune to some antigen other than SW virus.

TABLE 3.

Effect of heterologous antisera on a virus-antibody equilibrium.

Additional reagent	Antibody titre of filtrate
nil	2·44
normal rabbit serum 1	2·63
normal rabbit serum 2	2·53
rabbit anti-LEE* serum 1	2·50
rabbit anti-LEE* serum 2	2·53
fowl anti-LEE* serum	2·44

0·25 ml. SW virus (diluted 1:10) was mixed with 0·25 ml. rabbit anti-SW serum (diluted 1:20) in the presence of 0·50 ml. of the additional reagent. After 30 min. incubation the mixtures were passed through a gelatine-treated 'Millipore VM' membrane. The antibody titres are given in log units.

* The prototype strain of influenza B virus.

As the results in Table 3 show, the filtrates did not differ significantly among one another. It may be concluded therefore that the non-antibody fraction of sera does not influence the equilibrium established between the homologous reactants.

In the second experiment the mixtures of SW virus and anti-SW serum were set up in the presence of various other antigens. After the usual preincubation of 30 minutes, the preparations were filtered, and the concentrations of free antibody determined in the filtrate (Table 4).

TABLE 4.

Effect of heterologous antigens on a virus-antibody equilibrium.

Additional reagent	Antibody titre of filtrate
nil	2·44
LEE* virus $10^{9\cdot5}$ ID_{50}	2·53
Vaccinia virus $10^{7\cdot0}$ pfu	2·47
Rabbit pox virus $10^{8\cdot5}$ pfu	2·47

0·25 ml. SW virus (diluted 1:10) was mixed with 0·25 ml. rabbit anti-SW serum (diluted 1:20) in the presence of 0·50 ml. of the additional reagent. After 30 min. incubation the mixtures were passed through a gelatine-treated 'Millipore VM' membrane. The antibody titres are given in log units.

* The prototype strain of influenza B virus.

Once again, the answer is unequivocal: the same fraction of antibody remains uncombined whether the system contains the homologous antigen only, or also sundry heterologous ones.

Reproducibility and sensitivity of the technique.

With the technical details standardized, it remained to establish the reliability of the method. To this end each of us made up virus-antibody mixtures in sextuplicate, using two concentrations of virus. After 30 minutes' incubation at room temperature (20° C.) the mixtures were filtered, and antihaemagglutinin titres of the filtrates determined in duplicate. A test of identical design was performed with the same reagents two days later. The 96 titres so obtained were submitted to an analysis of variance. The main contrasts to be evaluated were the difference due to operators, to time of performance, to parallel tests with the two doses of virus, to replicate tests on the same virus-antibody mixture, and to antihaemagglutinin titrations.

The variance due to different doses of virus was highly significant (variance ratio $F_{(1, 88)} = 3078$, where the $P = 0{\cdot}001$ point lies at $11{\cdot}5$). This result is the same as can also be seen from the lower third of Fig. 2 – in the mid-range of binding the method is exquisitely sensitive to variation in the concentration of antigen. As far as reproducibility is concerned, however, the response to dose is of no relevance. The contrasts of interest, or more precisely, the cumulative errors introduced by their presence, are listed in Table 5.

The basic error, due to antihaemagglutinin titrations, accounts for two-thirds of the maximal error obtaining when all sources of variation are simultaneously operative. Indeed, in practice about as much would be gained by performing a single equilibrium filtration and then assaying the filtrate in duplicate, as by a very large number of filtration tests followed by single antihaemagglutinin titrations. (Duplicate antihaemagglutinin assays would reduce the error from ± 24 p.c. to ± 19 p.c., triplicates to ± 17 p.c.; an infinite number of filtration tests would bring it only to ± 16 p.c.)

Clearly, the accuracy of the method is limited by the accuracy of the antibody assay. And since antihaemagglutinin tests are more reproducible and more economical than any test for antiviral antibody, it may be concluded that the

TABLE 5.

Reproducibility of equilibrium filtration assays.

Source of variation	Cumulative standard deviation	
	$\log_{10}$ units	per cent.
Antihaemagglutinin test (basic error)	±0·063	±15·6
+ replicate filtration assays	±0·078	±19·7
+ parallel filtration assays	±0·078	±19·7
+ time of performance	±0·090	±23·0
+ operators	±0·093	±23·9

The estimates are based on an analysis of variance computed from the results of 96 tests.

method of ultrafiltration compares more than favourably with immunological techniques at present available to the virologist.

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METHODS IN IMMUNOCHEMISTRY OF VIRUSES

2. EVALUATION OF PARAMETERS FROM EQUILIBRIUM MEASUREMENTS

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(*Received for publication 29th October, 1961.*)

SUMMARY.

Principles governing the choice of variables and of the most informative range of concentrations for equilibrium measurements are discussed. The procedure found most suitable for work on viruses and their antibodies is equilibrium filtration of mixtures containing constant doses of antibody and variable amounts of virus. Measurements will be reliable and free from complications if the antigenic sites are far from saturation and at least nine-tenths of antibody are bound.

Two linear transforms of the law of mass action are derived; with the aid of either of these the equilibrium constant of the virus-antibody union and the number of antigenic sites per virus particle can be estimated simultaneously.

Statistical procedures appropriate to equilibrium measurements are given, together with rapid tests for checking the validity of all assumptions underlying the mathematical model.

The evaluation of parameters is demonstrated, in detail, on an example of influenza virus and an anti-influenzal rabbit serum.

Numerical tables needed for the expeditious solution of equilibrium equations are given in an Appendix.

INTRODUCTION.

In the preceding paper we have shown that equilibrium filtration provides a simple and unbiassed method for the study of equilibrium conditions in a virus-antibody system. All that has been said about filtration holds also for equilibrium dialysis and when the micro-equipment of Dubert (1959) is fitted with appropriate filters the two methods give identical results. However, unless the nature of the equilibrium is understood the design of experiments will be haphazard and the information obtained may well remain below what the technique could optimally yield.

Neither do the many ways in which equilibrium data are usually evaluated equally suit all experimental designs. Sometimes one, sometimes another transform will estimate a parameter with least uncertainty and often more than one will have to be used to do justice to more extensive sets of measurements. Bio-

logical material and the use of biological assays introduce a further difficulty. Unlike most physical techniques with linear – and usually small – error components, these tests have large variances and frequently non-normal error distributions. At the level of evaluation this raises the problem of adequately weighting the evidence and at the level of design the need to reckon with inherent restrictions. This paper is concerned with these two problems as well as with examining the assumptions on which equilibrium data are to be interpreted.

THEORETICAL.

Basic model.

Consider a system of V virus particles, each with s antigenic sites on its surface, and A antibody molecules per unit volume (1 ml.). According to the reaction

$$s + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} x \qquad \ldots\ldots \quad (1)$$

antigenic sites and antibody molecules combine to give antigen-antibody complexes, x in number per unit volume. The rate of association will be proportional to the concentrations of the free reactants and to the rate constant k_1. Thus

$$\frac{dx}{dt} = k_1 (sV - x)(A - x) \qquad \ldots\ldots \quad (2)$$

Dissociation will follow first order kinetics, being proportional to the concentration of antigen-antibody complexes and to the rate constant k_{-1}. Accordingly

$$-\frac{dx}{dt} = k_{-1} x \qquad \ldots\ldots \quad (3)$$

At equilibrium the two processes exactly balance and the rate of change of concentration of x equals zero:

$$\frac{dx}{dt} = 0 = k_1(sV - x)(A - x) - k_{-1} x \qquad \ldots\ldots \quad (4)$$

Writing $K = k_{-1}/k_1$, we arrive at the conventional expression of the mass law

$$K = \frac{(sV - x)(A - x)}{x} \qquad \ldots\ldots \quad (5)$$

It should be noted that K, defined as above, is in cgs units and differs by a factor of $1{\cdot}66 \times 10^{-21}$ from the conventional equilibrium constant expressed in molar terms.

Complications.

The derivation leading to Eq. (5) implies that there is no interaction between sites. This postulate will certainly hold in the region where x is small compared to sV, that is, far from saturation. Furthermore, antibody is treated

as univalent – this simplification, contrary to well-established experimental evidence, can be maintained only in the case of dilute systems where the mean free path of antibody is negligible compared to the average distance between viruses, that is, where collisions between antibody-laden virus particles occur with low probability only. Even if the latter condition were not strictly fulfilled, no substantial error can arise from the assumption of univalence as long as the system is far from saturation.

To first approximation, then, Eq. (5) will serve as descriptive model. It will be shown later how the validity of the two simplifying assumptions can be tested and, indeed, how such tests are incorporated into the routine estimation of parameters. Complications of another nature, issuing from the heterogeneity of antibody or of antigenic sites, will have effects that are usually not difficult to recognize; ways to assess their magnitude will be developed later.

Choice and range of the dependent variable.

The first decision taken on designing a method of assay is the choice of variables to be measured and of the range within which reliable estimation can be expected. In the virus-antibody system there is no choice open at present: we have no means of assessing directly the number of either free or occupied antigenic sites and thus the remaining variable, the concentration of free antibody, must be the variable measured.

This being the case, it is convenient to express the concentration of free and combined antibody as fractions of the original input. By this means the quantity x can be eliminated from the equations, and replaced by something directly measurable. We write then αA for the fraction of antibody which is present in the form of virus-antibody complexes, i.e., $x = \alpha A$. The fraction remaining free equals, obviously, $(1-\alpha)A$, since the two fractions must add up to the input concentration, A. In these terms Eq. (5) will read

$$K = \frac{(sV - \alpha A)\,(1-\alpha)A}{\alpha A} = (sV - \alpha A)\,\frac{1-\alpha}{\alpha} \quad \ldots\ldots \quad \ldots\ldots \quad (6)$$

Since the available types of assay measure free antibody, the quantities A and $(1-\alpha)A$ can be obtained by conventional titrations. Generally it is not A, the absolute concentration of antibody, that is determined but a serum titre. Serum titres are in operational terms and refer to that dilution of an antiserum which gives a specified endpoint-reaction when submitted to a particular, fully defined assay procedure. Serum titres differ from the absolute concentration of antibody by a constant factor, to be evaluated separately. Procedures used in the assay of antiviral sera are such that the logarithms of observed titres are normally distributed. Recognition of this fact is fundamental to the design of a valid method of evaluation but otherwise places no restriction on the range of concentrations within which the variables are to lie. The major restriction stems from the fact that only one component of the equilibrium system is being deter-

mined whereas Eq. (6) contains the ratio of this component and its complement, in the form of $(1-a)/a$. Evidently a is not estimated independently and as a consequence the ratios will not be distributed normally about their means. Intuitively, one would opt for as low values as possible for the experimentally determined fraction $(1-a)$ so that its complement could carry the lowest possible error. A more rigorous method of weighting will be considered in a separate communication dealing with the evaluation of heterogeneity.

Thus, when setting the optimal range of antibody concentrations in equilibrium experiments we have three criteria to govern us. First, the system must be far from saturation. Formally, this means that sV should be much larger than aA, and we may consider the arbitrary level of $sV \geqslant 10\,aA$ as satisfactory. Secondly, the probability of collisions between virus particles should be negligible within the time span of the experiment. Since the number of collisions per second is $8V^2r^2\sqrt{\pi kT/m}$ (where r and m stand for the radius and mass respectively of the virus particle, k is the Boltzmann constant, and T the absolute temperature), a concentration of $V = 2 \cdot 5.10^{11}$ in the case of influenza viruses will ensure that less than 10 p.c. of the virus particles will collide with another and remain bridged by antibody within half-an-hour at room temperature. This calculation takes into account the fact that only one-tenth of the collisions will also involve an antibody molecule on either of the virus particles and that the antigen-antibody union has an energy term of the order of e^{-10} at this temperature. Thirdly, the fraction of antibody remaining free should be much smaller than the fraction bound. Once more we may set the arbitrary limit at $0 \cdot 1 \geqslant (1-a)/a$.

Fortunately, at least with the viruses of influenza, these three criteria are not mutually exclusive. The limiting concentration of $2 \cdot 5.10^{11}$ virus particles corresponds to about 2.10^4 haemagglutinating doses and is rather more than an order of magnitude above the concentrations used in practice. Since each virus particle carries over a thousand antigenic sites (Fazekas de St.Groth and Webster, 1962a) this sets the input concentration of antibody at about 5.10^{13}, roughly corresponding to a 1:20 dilution of an hyperimmune serum. And since such sera have titres exceeding 1:1000, the estimation of free antibody should present no difficulty at an expected concentration of 1:200 in terms of the original serum.

If a system of the above constitution were at equilibrium, its equilibrium constant would be of the order of 10^{14} (of the order of 10^{-7} in molar terms) and the free energy of binding very close to 9 kcal/mole at 300° K. This region may be regarded as optimal for equilibrium measurements on influenza viruses, and the range can be readily extended to reagents characterized by equilibrium constants at least two orders of magnitude lower. If, however, binding between virus and antibody is weaker, all complications listed earlier are likely to be of importance and the evaluation of experimental data will require special care.

Choice of the independent variable.

There is no inherent reason for choosing either virus or antibody as independent variable in equilibrium tests nor, indeed, against varying them both. The first method (constant antibody, varying antigen) is known as the α-procedure in serology and the second (constant antigen, varying antibody) as the β-procedure. Treating both antigen and antibody as variables adds another dimension to the experiment and such tests go under the name of block or checkerboard tests, in the vernacular.

Unlike in the case of the dependent variable, circumstance does not compel to choose either virus or antibody as independent variable, and thus any of the three procedures could be considered. Constraints are self-imposed, and arise from the desire to avoid, at least initially, the complications discussed earlier. Accordingly, that procedure shall be chosen which gives the simplest functional relationship when the system is far from saturation, and in which the valency of antibody does not affect the estimation of parameters. This amounts to choosing the α-procedure, i.e., the concentration of virus as variable. Since we aim at having sV much larger than αA, the concentration of antibody will altogether disappear from Eq. (6) as $(sV - \alpha A)$ tends to sV. In the limit we have

$$K \to sV \frac{1-\alpha}{\alpha}, \text{ as } \alpha A/sV \to 0 \quad \ldots\ldots \quad \ldots\ldots \quad \ldots\ldots \quad (7)$$

It should be noted that Eq. (7) is the limiting form of Eq. (6) also if $A/K \to 0$, and this relationship will be used as a rapid test for checking whether measured equilibria lie in the desired range.

A test patterned on the β-procedure would be less convenient, both because the term sV cannot become negligible as long as the system is far from saturation, and because using A as independent variable would also retain α as its coefficient, thus giving prominence to the indirectly determined complement of the dependent variable. Any argument against the β-procedure holds, *a fortiori*, against block tests. Even then dealing with heterogeneous systems where three-dimensional tests would seem mandatory, alternative methods can be devised and shown to be more informative (Fazekas de St.Groth and Webster, 1962b).

Choice of functional form.

Equation (6), in the form stated, is unsuitable for the evaluation of the equilibrium constant K and the number of antigenic sites s, since the variables V and α are not separated. The solution of the equation as a quadratic in α does not help either as V then appears in three separate terms, and the functional relationship does not lend itself to simple statistical treatment. The immediate task therefore is to state Eq. (6) in forms that give manageable, preferably linear, plots and are amenable to conventional statistical treatment.

One such transform is obtained by dividing both sides of Eq. (6) by $(1-\alpha)K$, to give

$$\frac{1}{1-\alpha} = \frac{s}{K} \cdot \frac{V}{\alpha} - \frac{A}{K} \quad \ldots\ldots \quad \ldots\ldots \quad \ldots\ldots \quad (8)$$

This is the equation of a straight line when $1/(1-a)$ is plotted against V/a. The intercept with the ordinate will give $-A/K$, and hence K; whereas the slope, s/K, allows the evaluation of s.

Another suitable transform can be derived from Eq. (6) by dividing through by A:

$$\frac{K}{A} = \frac{sV(1-a)}{Aa} - (1-a)$$

and, after rearrangement

$$(1-a) = \frac{s}{A} \cdot \frac{V(1-a)}{a} - \frac{K}{A} \quad \ldots\ldots\ldots\ldots \quad (9)$$

This, again, defines a straight line relationship, by plotting $(1-a)$ against $V(1-a)/a$. The slope is s/A, and the intercept $-K/A$, allowing thus estimation of both K and s.

Both slopes (s/K in Eq. (8), and s/A in Eq. (9)) are expected to be small, of the order of 10^{-10} and 10^{-9}, respectively. It is convenient therefore to scale the equations by expressing the concentration of virus particles, V, as the product of an arbitrary concentration V_o, and a dilution factor, d, i.e., $V = V_o d$. By setting V_o as the concentration of virus particles in the stock preparation we shall not only bring the slopes close to unity, but also make the calculations operationally simplest since, in practice, concentrations of a particular reagent are usually thought of as dilutions, the necessary multiplication being carried out only when evaluating the results.

Examining the two transforms more closely we find that, with the limits set in the previous sections, the left-hand side of Eq. (8) will be equal or greater than 10, since $(1-a) \leqslant 0{\cdot}1$. Hence the first term of the right-hand side may be written, to good approximation, as sV/K, and the approximation will be the better the smaller the fraction of antibody remaining free. This would eliminate the error connected with the definition of a. However, if the condition $A/K \to 0$ is fulfilled, the curve will pass so close to the origin that no reliable estimate of the intercept can be expected. Since the equilibrium constant, K, is estimated from the intercept, and the value of K is required in turn to compute s, this transform cannot be used in the very range where experimental measurements are most reliable. And even if A/K is not very close to zero, the intercept with the ordinate is on the negative limb, whereas the observed values of the dependent variable lie at $+10$ or higher. As a consequence, K has to be estimated by considerable extrapolation and hence cannot be known with great accuracy. Since the error of K also boosts the relative error of s, the use of Eq. (8) is inadvisable under the conditions where equilibrium measurements are least likely to be complicated.

In Eq. (9) the independent variable appears in the form $(1-a)/a$ on the right-hand side. It is important, therefore, to ensure that experimental values of this ratio be as low as practicable in order to reduce the error of a. With this

proviso the two constants s and K can be estimated independently since, unlike in the slope-term of Eq. (8), the two are not confounded here. A further point in favour of Eq. (9) is that the extrapolation necessary to obtain the intercept, and thus the value of K, will always be at least an order of magnitude less than by the use of Eq. (8). This follows from the condition of $sV \geqslant 10A$. Some extrapolation, however, is unavoidable as the dependent variable cannot take negative values whereas the intercept of the regression line with the ordinate must always lie below zero.

There are circumstances when the combined use of Equations (8) and (9) is preferable to the use of Eq. (9) only. If $A \ll K$, Eq. (8) reduces to the form of Eq. (7), with the regression line passing through the origin, whereas the intercept of Eq. (9) will under these conditions approach $-\infty$. Here, by determining the slopes of both transforms, we obtain the value of s from Eq. (9), and then use this for computing K from the slope of Eq. (8).

Statistical.

Check on validity of assumptions.

Before entering on the evaluation of parameters, it is advisable to find out whether the variables lie within the limits set for uncomplicated tests. Two of the three criteria can be applied before the experiment, and the third by simple inspection of the results. Thus the concentration of virus particles and antibody molecules is known, and can always be so adjusted that interaction between antibody molecules and aggregation of virus particles remain negligible. If it is also found that most of the antibody had combined with antigen, the third criterion has been met as well. What remains to be checked is the ratio A/K, since on this depends essentially the method of evaluation.

To this end we consider the behaviour of Equations (8) and (9), for various values of the term A/K. When $A/K = 0$, Eq. (8) reduces to the form of Eq. (7), as already pointed out above. By writing $V_0 d$ for V, and rearranging, we obtain

$$\alpha = 1 - \frac{K}{sV_0} \cdot \frac{\alpha}{d} \qquad \text{when } A/K = 0 \quad \ldots\ldots \quad \ldots\ldots \quad \ldots\ldots \quad (10)$$

At the other extreme, when K/A becomes negligible, Eq. (9) gives

$$1 = \frac{A}{sV_0} \cdot \frac{\alpha}{d} \qquad \text{when } K/A = 0 \quad \ldots\ldots \quad \ldots\ldots \quad \ldots\ldots \quad (11)$$

that is, a constant from which the value of s can be derived directly. It is useful to establish the relationship for an intermediate reference value, say, when $A/K = 1$. Here

$$\frac{1-\alpha}{2-\alpha} = \frac{K}{sV_0} \cdot \frac{\alpha}{d} \qquad \text{when } A/K \equiv 1 \quad \ldots\ldots \quad \ldots\ldots \quad \ldots\ldots \quad (12)$$

Thus if α is plotted against α/d, Eq. (10) will give a straight line with negative slope. The intercept is at $+1$ and at the point of 50 p.c. binding ($\alpha = 0{\cdot}5$) $K = sV_0 d$. Both of these points lie very close to the experimentally testable range, and can therefore be determined with minimal extrapolation. When K is much smaller than A, and thus Eq. (11) is plotted on the same scale, a vertical straight line is obtained with no intercept on the ordinate, and passing through the abscissa at sV_0/A, that is, somewhere round the value of 10 or above. The intermediate case considered (Eq. (12)), has an intercept at $+1$ like Eq. (11), but turns gradually downwards and passes through the abscissa at the value of $0{\cdot}5\ sV_0/K$. A graphic representation of these three curves is given in Fig. 1.

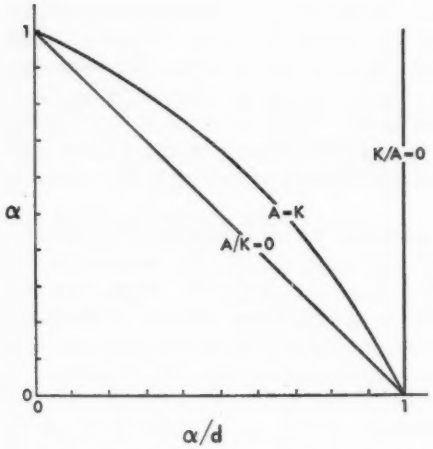

Fig. 1. Plot of Equations 10, 11 and 12. The scale of the abscissa is set by $sV_o = A + K$, for all three curves.

In general, the $(\alpha, \alpha/d)$ plot will have an intercept with the ordinate if $0 \leqslant A/K \leqslant 1$. If $A/K > 1$, the curves will not intercept the ordinate. Since the region where the alternatives are best distinguishable is precisely the region of greatest reliability (i.e. where α attains its highest values), the statistical method of evaluation shall be chosen by this criterion.

Thus, if on plotting α against α/d a line is obtained which passes through the point $(x = 0, y = 1)$ of the coordinate system, or through a point not significantly different from that, A/K is equal to or less than one, and hence a combination of Equations (8) and (9) will be required for best evaluation of the parameters. If the curve has no $(x = 0, y = 1)$ point, A/K is greater than one and Eq. (9) alone can be used. The complete procedure will be demonstrated below, on practical examples.

Statistical estimation.

Whichever of the functional forms is chosen, the same procedures will be needed for the evaluation of parameters. In either case a maximum likelihood estimate of a regression is required in order to obtain answers to the following questions. (1) Is the experimental information adequate, i.e., is the regression significant in terms of the error variance? (2) Is the underlying model valid, i.e., can the linearity of the regression be upheld? And if these two have been answered in the affirmative, (3) what is the slope of the regression line and with what degree of confidence can one assert its value? (4) What is the intercept and what is its error?

By plotting the experimental data according to the appropriate equations a presumptive answer can be obtained to all questions by simple inspection of the graph. Indeed, it is exceptional that one should have to rely on statistical treatment to decide whether a set of points can be fitted by a line and whether that line is straight or not. And a line fitted by eye will always give at least an approximate value for both slope and intercept. It is only when several such lines are to be compared, or when estimates free from subjective error are needed that statistical methods come into their own.

The association of the variables in Equations (8) and (9) is of the general form $y = a + bx$, that is, a straight line is to be fitted to the data. To this end a number of simple statistics have to be computed. First, the sums of the variables (Σx and Σy), the sums of their squares (Σx^2 and Σy^2), and the sum of their products (Σxy). From these are derived the intermediary statistics: $S_{xx} = \Sigma x^2 - (\Sigma x)^2/n$; $S_{yy} = \Sigma y^2 - (\Sigma y)^2/n$; $S_{xy} = \Sigma xy - \Sigma x\, \Sigma y/n$. By the use of these, objective answers can be furnished to each of the four questions listed above.

The variance of the regression is calculated as $V_1 = (S_{xy})^2/S_{xx}$ and the error variance as $V_2 = (S_{yy} - V_1)/(n-2)$. The ratio of these two $(V_1/V_2 = F_{(1,\, n-2)})$ can be looked up in a table of variance ratios, and if found significant, the first of the questions has been settled satisfactorily.

The best estimate of the slope is $b = S_{xy}/S_{xx}$, with an error of $\pm \sqrt{V_2 S_{xx}}$. The intercept is estimated as $a = \bar{y} - b\bar{x}$, and its standard error is $\pm \sqrt{V_2(1/n - \bar{x}^2/S_{xx})}$, where $\bar{x}$ and $\bar{y}$ stand for the means of x and y respectively ($\bar{x} = \Sigma x/n$, $\bar{y} = \Sigma y/n$). These are the results required to answer the third and fourth questions.

The second question would demand more cumbersome calculations by the orthodox methods, but we may propose here an alternative which, though less efficient, will still serve the purpose. Accordingly, two separate slopes and intercepts are calculated from the data, one for each half, and then a *t*-test performed on the results. If the two sets are found to differ significantly, either in respect to the value of the slopes or of the intercepts, the condition of linearity is contradicted, and the basic model is invalid. If the differences are not significant, question two has been answered in the affirmative.

TABLE 1.

Evaluation of an equilibrium filtration test: Experimental results.

Equal volumes of an hyperimmune rabbit serum against SW virus (diluted 1:10 in saline) and of dilutions of SW virus stock were mixed, kept at 20° C. for 30 minutes, and filtered through a gelatine-treated Millipore VM membrane. The antibody content of the original serum and of all filtrates was assayed in duplicate as antihaemagglutinin against 4 agglutinating doses of SW virus.

Dilution of virus d		Antibody titre of filtrate ($\log_{10}$ units/ml.)	Fraction of antibody free $\log(1-\alpha)$
1/6		1·90	−1·65
		2·00	−1·55
1/7		2·08	−1·47
		2·08	−1·47
1/8		2·29	−1·26
		2·20	−1·35
1/9		2·38	−1·17
		2·38	−1·17
1/10		2·50	−1·05
		2·47	−1·08
1/11		2·65	−0·90
		2·65	−0·90
1/12		2·68	−0·87
		2·68	−0·87
1/13		2·77	−0·78
		2·80	−0·75
1/14		2·80	−0·75
		2·80	−0·75
1/15		2·89	−0·66
		2·98	−0·57
1/20		3·01	−0·54
		3·01	−0·54
	filtered	3·55	
		3·55	
Controls (no virus)			≡0·00
	unfiltered	3·55	
		3·55	

PRACTICAL.

A worked example.

Finally, the method of estimating the parameters of virus-antibody union will be demonstrated on the results of an equilibrium filtration experiment.

A preparation of SW virus, containing $1{\cdot}63 \times 10^{11}$ particles per millilitre as found from electron microscopic counts by Sharp's method (1949), was diluted in saline, and 0·60 ml. volumes dispensed in small test tubes. To each of these tubes 0·60 ml. of a 1:10 dilution of hyperimmune anti-SW serum was added. As determined by electrodieresis (Fazekas de St.Groth and Webster, 1962c), the original serum contained $3{\cdot}27 \times 10^{14}$ antibody molecules per millilitre. After 30 minutes' incubation at 20° C. the virus-antiserum mixtures were filtered through gelatine-treated Millipore VM membranes, as described in the companion paper (Fazekas de St.Groth and Webster, 1961). All filtrates as well as the original serum were assayed for antibody in duplicate, with the results shown in Table 1.

The first column gives the dilutions of virus and includes the controls (the standard dose of antiserum mixed with saline instead of virus, and tested for antibody both before and after filtration). The second column shows the $\log_{10}$ titres of the filtrates, and the third the fraction of antibody passing the filter. The latter values were obtained by subtracting the mean control titres (3·55) from each of the titres. From these figures, with the aid of the tables given in the Appendix, the values α/d and α are worked out and plotted (Fig. 2).

Evidently, the curve fitted to the points does not intercept the ordinate at $+1$, and hence we conclude that $A/K > 1$. On this evidence Eq. (9) is to be used for evaluation of the parameters.

Referring once more to the tables of the Appendix, the appropriate variables are listed (Table 2) and plotted (Fig. 3).

From inspection of the curve there can be little doubt that the regression is significant, and that the experimental points represented by dots (the range where $(1-\alpha)/\alpha < 0{\cdot}1$) are well fitted by a straight line. These are the only points from which the parameters will be estimated, but for the sake of completeness the rest of the data is also shown in the Figure by circles. In effect, the straight line fitted over the 0 to 0·1 range appears reasonable up to the value of $(1-\alpha) = 0{\cdot}20$. The last four points show definite curvature towards the ordinate and also wider scatter, as could be expected from the emergence of complications in this region and from the increasing uncertainty of the estimate of α.

TABLE 2.

Evaluation of an equilibrium filtration test: fitting of equation $(1-\alpha) = \frac{sV_0}{A}\left(d\frac{1-\alpha}{\alpha}\right) - \frac{K}{A}$

$x = \left(d\frac{1-\alpha}{\alpha}\right)$	$y = (1-\alpha)$
·0037	·022
·0048	·028
·0050	·034
·0050	·034
·0073	·055
·0059	·045
·0081	·068
·0081	·068
·0098	·089
·0091	·083

The transformed variables correspond to the first ten entries of Table 1.

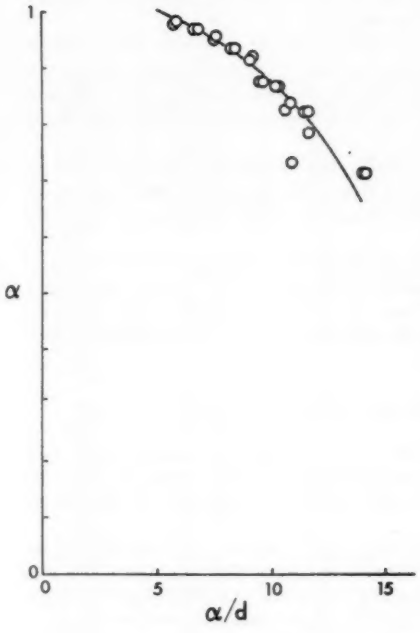

Fig. 2. Choice of variables. The screening plot (α, α/d) is based on the data of Table 1.

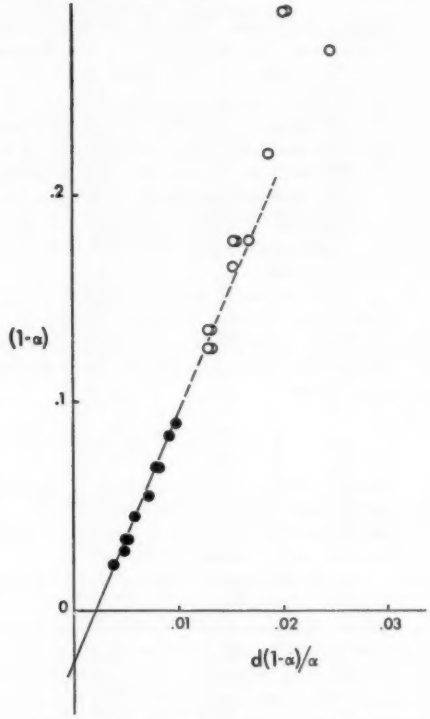

Fig. 3. Estimation of parameters by Equation 9. The data of Table 1 falling in the most reliable range are represented by dots, the rest by circles. The straight line is fitted by eye, to the first ten entries of Table 1.

The line fitted by eye has a slope of 12, and this is a rough estimate of sV_0/A. The intercept is at $-0{\cdot}025$, indicating that A is about 40-times as large as K ($K/A \doteqdot 0{\cdot}025$). To arrive at more reliable values, the computations set down in the statistical section are to be performed. Accordingly

$$\bar{x} = 0{\cdot}00668 \qquad \Sigma x^2 = \quad 0{\cdot}00048498$$
$$\bar{y} = 0{\cdot}0526 \qquad -(\Sigma x)^2/n = -0{\cdot}000446224$$
$$S_{xx} = \quad 0{\cdot}000038756$$

$$\Sigma xy = \quad 0{\cdot}0039519 \qquad \Sigma y^2 = \quad 0{\cdot}032688$$
$$-\Sigma x \Sigma y/n = -0{\cdot}00351368 \qquad -(\Sigma y)^2 n = -0{\cdot}0276676$$
$$S_{xy} = \quad 0{\cdot}00043822 \qquad S_{yy} = \quad 0{\cdot}0050204$$

From these statistics we obtain further

$$V_1 = (S_{xy})^2/S_{xx} = 0{\cdot}0049550$$
$$V_2 = (S_{yy} - V_1)/(n-2) = 0{\cdot}000008175.$$

The ratio of these two, $F_{(1,\,8)} = V_1/V_2 = 606{\cdot}12$, and reference to a variance ratio table

(Fisher and Yates, 1953) shows that a value as high as this would be obtained by chance in less than 1 in 1000 cases, i.e., the regression is significant.

The value of the slope is $b = S_{xy}/S_{xx} = 11{\cdot}307$, and its standard error equals $\pm \sqrt{V_2/S_{xx}} = 0{\cdot}459$, or about $\pm$ 4 p.c. Multiplying the error term by Student's t-value for $(n-2)$ degrees of freedom at the 5 p.c. level of probability, 2·306, we obtain $\pm$ 1·058, and can thus assert that the best estimate of the slope is 11·3, and it will lie within the limits of 10·25 and 12·36 with 95 p.c. probability.

The intercept is found as $a = \bar{y} - b\bar{x} = -0{\cdot}02293$, and carries the standard error of $\pm \sqrt{V_2(1/n - \bar{x}^2/S_{xx})} = 0{\cdot}00321$. Using the same t-value as above, we arrive at the 95 p.c. confidence interval of 0·0155 to 0·0303, within which the value of a (i.e., of the ratio K/A) will fall.

If an objective test of linearity be required, the slope and intercept for the first and second five entries of Table 2 may be calculated separately. The results show that $b_1 = 9{\cdot}38 \pm 0{\cdot}805$, $a_1 = -0{\cdot}0138 \pm 0{\cdot}00425$, and $b_2 = 11{\cdot}52 \pm 0{\cdot}590$, $a_2 = -0{\cdot}0239 \pm 0{\cdot}00521$. To judge whether they are significantly different, a t-test is performed on each pair. Thus

$$t_{(6)} = \frac{b_2 - b_1}{\sqrt{e^2_2 + e^2_1}} = 2{\cdot}15 \text{ and } t_{(6)} = \frac{a_2 - a_1}{\sqrt{e^2_2 + e^2_1}} = 1{\cdot}50.$$

where e stands for the appropriate standard errors. Reference to the t-tables shows that neither the slopes nor the intercepts differ from each other significantly, confirming that our first impression from Fig. 3 was correct, and the model used in the estimation of parameters has not been contradicted.

Since the original virus preparation contained $1{\cdot}63 \times 10^{11}$ particles, and was diluted by an equal volume of serum, $V_o = 8{\cdot}15 \times 10^{10}$. The concentration of the antiserum in the test is equivalent to a 1:20 dilution of the original serum, and thus $A = 1{\cdot}635 \times 10^{13}$. From these values the number of antigenic sites per virus particle can be obtained since $b = sV_o/A = 11{\cdot}307$, and hence $s = 2268$, with fiducial limits of 2056, 2480. Similarly, $a = -K/A = -0{\cdot}02293$, and hence $K = 3{\cdot}75 \times 10^{11}$, with limits of $2{\cdot}53 \times 10^{11}$, $4{\cdot}95 \times 10^{11}$.

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APPENDIX.

Numerical Tables for the Solution of Equilibrium Equations.

$1-\alpha$	$\frac{1}{1-\alpha}$	$\frac{1-\alpha}{\alpha}$	$\alpha(1-\alpha)$	$\frac{\alpha}{1-\alpha}$	$\frac{1}{\alpha}$	α
·001	1000·000	·0010	·00099	999·0000	1·001	·999
·002	500·000	·0020	·00199	499·0000	1·002	·998
·003	333·333	·0030	·00299	332·3333	1·003	·997
·004	250·000	·0040	·00398	249·0000	1·004	·996
·005	200·000	·0050	·00497	199·0000	1·005	·995
·006	166·666	·0060	·00596	165·6666	1·006	·994
·007	142·857	·0070	·00695	141·8571	1·007	·993
·008	125·000	·0080	·00793	124·0000	1·008	·992
·009	111·111	·0090	·00891	110·1111	1·009	·991
·010	100·000	·0101	·00990	99·0000	1·010	·990
·011	90·909	·0111	·01087	89·9090	1·011	·989
·012	83·333	·0121	·01185	82·3333	1·012	·988
·013	76·923	·0131	·01283	75·9230	1·013	·987
·014	71·428	·0141	·01380	70·4285	1·014	·986
·015	66·666	·0152	·01477	65·6666	1·015	·985
·016	62·500	·0162	·01574	61·5000	1·016	·984
·017	58·823	·0172	·01671	57·8235	1·017	·983
·018	55·555	·0183	·01767	54·5555	1·018	·982
·019	52·631	·0193	·01863	51·6315	1·019	·981
·020	50·000	·0204	·01960	49·0000	1·020	·980
·021	47·619	·0214	·02055	46·6190	1·021	·979
·022	45·454	·0224	·02151	44·4545	1·022	·978
·023	43·478	·0235	·02247	42·4782	1·023	·977
·024	41·666	·0245	·02342	40·6666	1·024	·976
·025	40·000	·0256	·02437	39·0000	1·025	·975
·026	38·461	·0266	·02532	37·4615	1·026	·974
·027	37·037	·0277	·02627	36·0370	1·027	·973
·028	35·714	·0288	·02721	34·7142	1·028	·972
·029	34·482	·0298	·02815	33·4827	1·029	·971
·030	33·333	·0309	·02910	32·3333	1·030	·970
·031	32·258	·0319	·03003	31·2580	1·031	·969
·032	31·250	·0330	·03097	30·2500	1·033	·968
·033	30·303	·0341	·03191	29·3030	1·034	·967
·034	29·411	·0351	·03284	28·4117	1·035	·966
·035	28·571	·0362	·03377	27·5714	1·036	·965
·036	27·777	·0373	·03470	26·7777	1·037	·964
·037	27·027	·0384	·03563	26·0270	1·038	·963
·038	26·315	·0395	·03655	25·3157	1·039	·962
·039	25·641	·0405	·03747	24·6410	1·040	·961
·040	25·000	·0416	·03840	24·0000	1·041	·960
·041	24·390	·0427	·03931	23·3902	1·042	·959
·042	23·809	·0438	·04023	22·8095	1·043	·958
·043	23·255	·0449	·04115	22·2558	1·044	·957
·044	22·727	·0460	·04206	21·7272	1·046	·956
·045	22·222	·0471	·04297	21·2222	1·047	·955
·046	21·739	·0482	·04388	20·7391	1·048	·954
·047	21·276	·0493	·04479	20·2765	1·049	·953
·048	20·833	·0504	·04569	19·8333	1·050	·952
·049	20·408	·0515	·04659	19·4081	1·051	·951
·050	20·000	·0526	·04750	19·0000	1·052	·950
·051	19·607	·0537	·04839	18·6078	1·053	·949
·052	19·230	·0548	·04929	18·2307	1·054	·948
·053	18·867	·0559	·05019	17·8679	1·055	·947
·054	18·518	·0570	·05108	17·5185	1·057	·946
·055	18·181	·0582	·05197	17·1818	1·058	·945
·056	17·857	·0593	·05286	16·8571	1·059	·944
·057	17·543	·0604	·05375	16·5438	1·060	·943
·058	17·241	·0615	·05463	16·2413	1·061	·942
·059	16·949	·0626	·05551	15·9491	1·062	·941
·060	16·666	·0638	·05640	15·6666	1·063	·940
·061	16·393	·0649	·05727	15·3934	1·064	·939
·062	16·129	·0660	·05815	15·1290	1·066	·938
·063	15·873	·0672	·05903	14·8730	1·067	·937
·064	15·625	·0683	·05990	14·6250	1·068	·936
·065	15·384	·0695	·06077	14·3846	1·069	·935
·066	15·151	·0706	·06164	14·1515	1·070	·934
·067	14·925	·0718	·06251	13·9253	1·071	·933
·068	14·705	·0729	·06337	13·7058	1·072	·932
·069	14·492	·0741	·06423	13·4927	1·074	·931
·070	14·285	·0752	·06510	13·2857	1·075	·930
α	$\frac{1}{\alpha}$	$\frac{\alpha}{1-\alpha}$	$\alpha(1-\alpha)$	$\frac{1-\alpha}{\alpha}$	$\frac{1}{1-\alpha}$	$1-\alpha$

$1-\alpha$	$\frac{1}{1-\alpha}$	$\frac{1-\alpha}{\alpha}$	$\alpha(1-\alpha)$	$\frac{\alpha}{1-\alpha}$	$\frac{1}{\alpha}$	α
·071	14·084	·0764	·06595	13·0845	1·076	·929
·072	13·888	·0775	·06681	12·8888	1·077	·928
·073	13·698	·0787	·06767	12·6986	1·078	·927
·074	13·513	·0799	·06852	12·5135	1·079	·926
·075	13·333	·0810	·06937	12·3333	1·081	·925
·076	13·157	·0822	·07022	12·1578	1·082	·924
·077	12·987	·0834	·07107	11·9870	1·083	·923
·078	12·820	·0845	·07191	11·8205	1·084	·922
·079	12·658	·0857	·07275	11·6582	1·085	·921
·080	12·500	·0869	·07360	11·5000	1·086	·920
·081	12·345	·0881	·07443	11·3456	1·088	·919
·082	12·195	·0893	·07527	11·1951	1·089	·918
·083	12·048	·0905	·07611	11·0481	1·090	·917
·084	11·904	·0917	·07694	10·9047	1·091	·916
·085	11·764	·0928	·07777	10·7647	1·092	·915
·086	11·627	·0940	·07860	10·6279	1·094	·914
·087	11·494	·0952	·07943	10·4942	1·095	·913
·088	11·363	·0964	·08025	10·3636	1·096	·912
·089	11·235	·0976	·08107	10·2359	1·097	·911
·090	11·111	·0989	·08190	10·1111	1·098	·910
·091	10·989	·1001	·08271	9·9890	1·100	·909
·092	10·869	·1013	·08353	9·8695	1·101	·908
·093	10·752	·1025	·08435	9·7526	1·102	·907
·094	10·638	·1037	·08516	9·6382	1·103	·906
·095	10·526	·1049	·08597	9·5263	1·104	·905
·096	10·416	·1061	·08678	9·4166	1·106	·904
·097	10·309	·1074	·08759	9·3092	1·107	·903
·098	10·204	·1086	·08839	9·2040	1·108	·902
·099	10·101	·1098	·08919	9·1010	1·109	·901
·100	10·000	·1111	·09000	9·0000	1·111	·900
·101	9·900	·1123	·09079	8·9009	1·112	·899
·102	9·803	·1135	·09159	8·8039	1·113	·898
·103	9·708	·1148	·09239	8·7087	1·114	·897
·104	9·615	·1160	·09318	8·6153	1·116	·896
·105	9·523	·1173	·09397	8·5238	1·117	·895
·106	9·433	·1185	·09476	8·4339	1·118	·894
·107	9·345	·1198	·09555	8·3457	1·119	·893
·108	9·259	·1210	·09633	8·2592	1·121	·892
·109	9·174	·1223	·09711	8·1743	1·122	·891
·110	9·090	·1235	·09790	8·0909	1·123	·890
·111	9·009	·1248	·09867	8·0090	1·124	·889
·112	8·928	·1261	·09945	7·9285	1·126	·888
·113	8·849	·1273	·10023	7·8495	1·127	·887
·114	8·771	·1286	·10100	7·7719	1·128	·886
·115	8·695	·1299	·10177	7·6956	1·129	·885
·116	8·620	·1312	·10254	7·6206	1·131	·884
·117	8·547	·1325	·10331	7·5470	1·132	·883
·118	8·474	·1337	·10407	7·4745	1·133	·882
·119	8·403	·1350	·10483	7·4033	1·135	·881
·120	8·333	·1363	·10560	7·3333	1·136	·880
·121	8·264	·1376	·10635	7·2644	1·137	·879
·122	8·196	·1389	·10711	7·1967	1·138	·878
·123	8·130	·1402	·10787	7·1300	1·140	·877
·124	8·064	·1415	·10862	7·0645	1·141	·876
·125	8·000	·1428	·10937	7·0000	1·142	·875
·126	7·936	·1441	·11012	6·9365	1·144	·874
·127	7·874	·1454	·11087	6·8740	1·145	·873
·128	7·812	·1467	·11161	6·8125	1·146	·872
·129	7·751	·1481	·11235	6·7519	1·148	·871
·130	7·692	·1494	·11310	6·6923	1·149	·970
·131	7·633	·1507	·11383	6·6335	1·150	·869
·132	7·575	·1520	·11457	6·5757	1·152	·868
·133	7·518	·1534	·11531	6·5187	1·153	·867
·134	7·462	·1547	·11604	6·4626	1·154	·866
·135	7·407	·1560	·11677	6·4074	1·156	·865
·136	7·352	·1574	·11750	6·3529	1·157	·864
·137	7·299	·1587	·11823	6·2992	1·158	·863
·138	7·246	·1600	·11895	6·2463	1·160	·862
·139	7·194	·1614	·11967	6·1942	1·161	·861
·140	7·142	·1627	·12040	6·1428	1·162	·860
α	$\frac{1}{\alpha}$	$\frac{\alpha}{1-\alpha}$	$\alpha(1-\alpha)$	$\frac{1-\alpha}{\alpha}$	$\frac{1}{1-\alpha}$	$1-\alpha$

$1-\alpha$	$\frac{1}{1-\alpha}$	$\frac{1-\alpha}{\alpha}$	$\alpha(1-\alpha)$	$\frac{\alpha}{1-\alpha}$	$\frac{1}{\alpha}$	α
·141	7·092	·1641	·12111	6·0921	1·164	·859
·142	7·042	·1655	·12183	6·0422	1·165	·858
·143	6·993	·1668	·12255	5·9930	1·166	·857
·144	6·944	·1682	·12326	5·9444	1·168	·856
·145	6·896	·1695	·12397	5·8965	1·169	·855
·146	6·849	·1709	·12468	5·8493	1·170	·854
·147	6·802	·1723	·12539	5·8027	1·172	·853
·148	6·756	·1737	·12609	5·7567	1·173	·852
·149	6·711	·1750	·12679	5·7114	1·175	·851
·150	6·666	·1764	·12750	5·6666	1·176	·850
·151	6·622	·1778	·12819	5·6225	1·177	·849
·152	6·578	·1792	·12889	5·5789	1·179	·848
·153	6·535	·1806	·12959	5·5359	1·180	·847
·154	6·493	·1820	·13028	5·4935	1·182	·846
·155	6·451	·1834	·13097	5·4516	1·183	·845
·156	6·410	·1848	·13166	5·4102	1·184	·844
·157	6·369	·1862	·13235	5·3694	1·186	·843
·158	6·329	·1876	·13303	5·3291	1·187	·842
·159	6·289	·1890	·13371	5·2893	1·189	·841
·160	6·250	·1904	·13440	5·2500	1·190	·840
·161	6·211	·1918	·13507	5·2111	1·191	·839
·162	6·172	·1933	·13575	5·1728	1·193	·838
·163	6·134	·1947	·13643	5·1349	1·194	·837
·164	6·097	·1961	·13710	5·0975	1·196	·836
·165	6·060	·1976	·13777	5·0606	1·197	·835
·166	6·024	·1990	·13844	5·0240	1·199	·834
·167	5·988	·2004	·13911	4·9880	1·200	·833
·168	5·952	·2019	·13977	4·9523	1·201	·832
·169	5·917	·2033	·14043	4·9171	1·203	·831
·170	5·882	·2048	·14110	4·8823	1·204	·830
·171	5·847	·2062	·14175	4·8479	1·206	·829
·172	5·813	·2077	·14241	4·8139	1·207	·828
·173	5·780	·2091	·14307	4·7803	1·209	·827
·174	5·747	·2106	·14372	4·7471	1·210	·826
·175	5·714	·2121	·14437	4·7142	1·212	·825
·176	5·681	·2135	·14502	4·6818	1·213	·824
·177	5·649	·2150	·14567	4·6497	1·215	·823
·178	5·617	·2165	·14631	4·6179	1·216	·822
·179	5·586	·2180	·14695	4·5865	1·218	·821
·180	5·555	·2195	·14760	4·5555	1·219	·820
·181	5·524	·2210	·14823	4·5248	1·221	·819
·182	5·494	·2224	·14887	4·4945	1·222	·818
·183	5·464	·2239	·14951	4·4644	1·223	·817
·184	5·434	·2254	·15014	4·4347	1·225	·816
·185	5·405	·2269	·15077	4·4054	1·226	·815
·186	5·376	·2285	·15140	4·3763	1·228	·814
·187	5·347	·2300	·15203	4·3475	1·230	·813
·188	5·319	·2315	·15265	4·3191	1·231	·812
·189	5·291	·2330	·15327	4·2910	1·233	·811
·190	5·263	·2345	·15390	4·2631	1·234	·810
·191	5·235	·2360	·15451	4·2356	1·236	·809
·192	5·208	·2376	·15513	4·2083	1·237	·808
·193	5·181	·2391	·15575	4·1813	1·239	·807
·194	5·154	·2406	·15636	4·1546	1·240	·806
·195	5·128	·2422	·15697	4·1282	1·242	·805
·196	5·102	·2437	·15758	4·1020	1·243	·804
·197	5·076	·2453	·15819	4·0761	1·245	·803
·198	5·050	·2468	·15879	4·0505	1·246	·802
·199	5·025	·2484	·15939	4·0251	1·248	·801
·200	5·000	·2500	·16000	4·0000	1·250	·800
·201	4·975	·2515	·16059	3·9751	1·251	·799
·202	4·950	·2531	·16119	3·9504	1·253	·798
·203	4·926	·2547	·16179	3·9261	1·254	·797
·204	4·901	·2562	·16238	3·9019	1·256	·796
·205	4·878	·2578	·16297	3·8780	1·257	·795
·206	4·854	·2594	·16356	3·8543	1·259	·794
·207	4·830	·2610	·16415	3·8309	1·261	·793
·208	4·807	·2626	·16473	3·8076	1·262	·792
·209	4·784	·2642	·16531	3·7846	1·264	·791
·210	4·761	·2658	·16590	3·7619	1·265	·790
α	$\frac{1}{\alpha}$	$\frac{\alpha}{1-\alpha}$	$\alpha(1-\alpha)$	$\frac{1-\alpha}{\alpha}$	$\frac{1}{1-\alpha}$	$1-\alpha$

$1-\alpha$	$\frac{1}{1-\alpha}$	$\frac{1-\alpha}{\alpha}$	$\alpha(1-\alpha)$	$\frac{\alpha}{1-\alpha}$	$\frac{1}{\alpha}$	α
·211	4·739	·2674	·16647	3·7393	1·267	·789
·212	4·716	·2690	·16705	3·7169	1·269	·788
·213	4·694	·2706	·16763	3·6948	1·270	·787
·214	4·672	·2722	·16820	3·6728	1·272	·786
·215	4·651	·2738	·16877	3·6511	1·273	·785
·216	4·629	·2755	·16934	3·6296	1·275	·784
·217	4·608	·2771	·16991	3·6082	1·277	·783
·218	4·587	·2787	·17047	3·5871	1·278	·782
·219	4·566	·2804	·17103	3·5662	1·280	·781
·220	4·545	·2820	·17160	3·5454	1·282	·780
·221	4·524	·2836	·17215	3·5248	1·283	·779
·222	4·504	·2853	·17271	3·5045	1·285	·778
·223	4·484	·2870	·17327	3·4843	1·287	·777
·224	4·464	·2886	·17382	3·4642	1·288	·776
·225	4·444	·2903	·17437	3·4444	1·290	·775
·226	4·424	·2919	·17492	3·4247	1·291	·774
·227	4·405	·2936	·17547	3·4052	1·293	·773
·228	4·385	·2953	·17601	3·3859	1·295	·772
·229	4·366	·2970	·17655	3·3668	1·297	·771
·230	4·347	·2987	·17710	3·3478	1·298	·770
·231	4·329	·3003	·17763	3·3290	1·300	·769
·232	4·310	·3020	·17817	3·3103	1·302	·768
·233	4·291	·3037	·17871	3·2918	1·303	·767
·234	4·273	·3054	·17924	3·2735	1·305	·766
·235	4·255	·3071	·17977	3·2553	1·307	·765
·236	4·237	·3089	·18030	3·2372	1·308	·764
·237	4·219	·3106	·18083	3·2194	1·310	·763
·238	4·201	·3123	·18135	3·2016	1·312	·762
·239	4·184	·3140	·18187	3·1841	1·314	·761
·240	4·166	·3157	·18240	3·1666	1·315	·760
·241	4·149	·3175	·18291	3·1493	1·317	·759
·242	4·132	·3192	·18343	3·1322	1·319	·758
·243	4·115	·3210	·18395	3·1152	1·321	·757
·244	4·098	·3227	·18446	3·0983	1·322	·756
·245	4·081	·3245	·18497	3·0816	1·324	·755
·246	4·065	·3262	·18548	3·0650	1·326	·754
·247	4·048	·3280	·18599	3·0485	1·328	·753
·248	4·032	·3297	·18649	3·0322	1·329	·752
·249	4·016	·3315	·18699	3·0160	1·331	·751
·250	4·000	·3333	·18750	3·0000	1·333	·750
·251	3·984	·3351	·18799	2·9840	1·335	·749
·252	3·968	·3368	·18849	2·9682	1·336	·748
·253	3·952	·3386	·18899	2·9525	1·338	·747
·254	3·937	·3404	·18948	2·9370	1·340	·746
·255	3·921	·3422	·18997	2·9215	1·342	·745
·256	3·906	·3440	·19046	2·9062	1·344	·744
·257	3·891	·3458	·19095	2·8910	1·345	·743
·258	3·875	·3477	·19143	2·8759	1·347	·742
·259	3·861	·3495	·19191	2·8610	1·349	·741
·260	3·846	·3513	·19240	2·8461	1·351	·740
·261	3·831	·3531	·19287	2·8314	1·353	·739
·262	2·816	·3550	·19335	2·8167	1·355	·738
·263	3·802	·3568	·19383	2·8022	1·356	·737
·264	3·787	·3586	·19430	2·7878	1·358	·736
·265	3·773	·3605	·19477	2·7735	1·360	·735
·266	3·759	·3623	·19524	2·7593	1·362	·734
·267	3·745	·3642	·19571	2·7453	1·364	·733
·268	3·731	·3661	·19617	2·7313	1·366	·732
·269	3·717	·3679	·19663	2·7174	1·367	·731
·270	3·703	·3698	·19710	2·7037	1·369	·730
·271	3·690	·3717	·19755	2·6900	1·371	·729
·272	3·676	·3736	·19801	2·6764	1·373	·728
·273	3·663	·3755	·19847	2·6630	1·375	·727
·274	3·649	·3774	·19892	2·6496	1·377	·726
·275	3·636	·3793	·19937	2·6363	1·379	·725
·276	3·623	·3812	·19982	2·6231	1·381	·724
·277	3·610	·3831	·20027	2·6101	1·383	·723
·278	3·597	·3850	·20071	2·5971	1·385	·722
·279	3·584	·3869	·20115	2·5842	1·386	·721
·280	3·571	·3888	·20160	2·5714	1·388	·720
α	$\frac{1}{\alpha}$	$\frac{\alpha}{1-\alpha}$	$\alpha(1-\alpha)$	$\frac{1-\alpha}{\alpha}$	$\frac{1}{1-\alpha}$	$1-\alpha$

$1-\alpha$	$\frac{1}{1-\alpha}$	$\frac{1-\alpha}{\alpha}$	$\alpha(1-\alpha)$	$\frac{\alpha}{1-\alpha}$	$\frac{1}{\alpha}$	α
·281	3·558	·3908	·20203	2·5587	1·390	·719
·282	3·546	·3927	·20247	2·5460	1·392	·718
·283	3·533	·3947	·20291	2·5335	1·394	·717
·284	3·521	·3966	·20334	2·5211	1·396	·716
·285	3·508	·3986	·20377	2·5087	1·398	·715
·286	3·496	·4005	·20420	2·4965	1·400	·714
·287	3·484	·4025	·20463	2·4843	1·402	·713
·288	3·472	·4044	·20505	2·4722	1·404	·712
·289	3·460	·4064	·20547	2·4602	1·406	·711
·290	3·448	·4084	·20590	2·4482	1·408	·710
·291	3·436	·4104	·20631	2·4364	1·410	·709
·292	3·424	·4124	·20673	2·4246	1·412	·708
·293	3·412	·4144	·20715	2·4129	1·414	·707
·294	3·401	·4164	·20756	2·4013	1·416	·706
·295	3·389	·4184	·20797	2·3898	1·418	·705
·296	3·378	·4204	·20838	2·3783	1·420	·704
·297	3·367	·4224	·20879	2·3670	1·422	·703
·298	3·355	·4245	·20919	2·3557	1·424	·702
·299	3·344	·4265	·20959	2·3444	1·426	·701
·300	3·333	·4285	·21000	2·3333	1·428	·700
·301	3·322	·4306	·21039	2·3222	1·430	·699
·302	3·311	·4326	·21079	2·3112	1·432	·698
·303	3·300	·4347	·21119	2·3003	1·434	·697
·304	3·289	·4367	·21158	2·2894	1·436	·696
·305	3·278	·4388	·21197	2·2786	1·438	·695
·306	3·267	·4409	·21236	2·2679	1·440	·694
·307	3·257	·4430	·21275	2·2573	1·443	·693
·308	3·246	·4450	·21313	2·2467	1·445	·692
·309	3·236	·4471	·21351	2·2362	1·447	·691
·310	3·225	·4492	·21390	2·2258	1·449	·690
·311	3·215	·4513	·21427	2·2154	1·451	·689
·312	3·205	·4534	·21465	2·2051	1·453	·688
·313	3·194	·4566	·21503	2·1948	1·455	·687
·314	3·184	·4577	·21540	2·1847	1·457	·686
·315	3·174	·4598	·21577	2·1746	1·459	·685
·316	3·164	·4619	·21614	2·1645	1·461	·684
·317	3·154	·4641	·21651	2·1545	1·464	·683
·318	3·144	·4662	·21687	2·1446	1·466	·682
·319	3·134	·4684	·21723	2·1347	1·468	·681
·320	3·125	·4705	·21760	2·1250	1·470	·680
·321	3·115	·4727	·21795	2·1152	1·472	·679
·322	3·105	·4749	·21831	2·1055	1·474	·678
·323	3·095	·4771	·21867	2·0959	1·477	·677
·324	3·086	·4792	·21902	2·0864	1·479	·676
·325	3·076	·4814	·21937	2·0769	1·481	·675
·326	3·067	·4836	·21972	2·0674	1·483	·674
·327	3·058	·4858	·22007	2·0581	1·485	·673
·328	3·048	·4880	·22041	2·0487	1·488	·672
·329	3·039	·4903	·22075	2·0395	1·490	·671
·330	3·030	·4925	·22110	2·0303	1·492	·670
·331	3·021	·4947	·22143	2·0211	1·494	·669
·332	3·012	·4970	·22177	2·0120	1·497	·668
·333	3·003	·4992	·22211	2·0030	1·499	·667
·334	2·994	·5015	·22244	1·9940	1·501	·666
·335	2·985	·5037	·22277	1·9850	1·503	·665
·336	2·976	·5060	·22310	1·9761	1·506	·664
·337	2·967	·5082	·22343	1·9673	1·508	·663
·338	2·958	·5105	·22375	1·9585	1·510	·662
·339	2·949	·5128	·22407	1·9498	1·512	·661
·340	2·941	·5151	·22440	1·9411	1·515	·660
·341	2·932	·5174	·22471	1·9325	1·517	·659
·342	2·923	·5197	·22503	1·9239	1·519	·658
·343	2·915	·5220	·22535	1·9154	1·522	·657
·344	2·906	·5243	·22566	1·9069	1·524	·656
·345	2·898	·5267	·22597	1·8985	1·526	·655
·346	2·890	·5290	·22628	1·8901	1·529	·654
·347	2·881	·5313	·22659	1·8818	1·531	·653
·348	2·873	·5337	·22689	1·8735	1·533	·652
·349	2·865	·5360	·22719	1·8653	1·536	·651
·350	2·857	·5384	·22750	1·8571	1·538	·650
α	$\frac{1}{\alpha}$	$\frac{\alpha}{1-\alpha}$	$\alpha(1-\alpha)$	$\frac{1-\alpha}{\alpha}$	$\frac{1}{1-\alpha}$	$1-\alpha$

$1-\alpha$	$\frac{1}{1-\alpha}$	$\frac{1-\alpha}{\alpha}$	$\alpha(1-\alpha)$	$\frac{\alpha}{1-\alpha}$	$\frac{1}{\alpha}$	α
·351	2·849	·5408	·22779	1·8490	1·540	·649
·352	2·840	·5432	·22809	1·8409	1·543	·648
·353	2·832	·5455	·22839	1·8328	1·545	·647
·354	2·824	·5479	·22868	1·8248	1·547	·646
·355	2·816	·5503	·22897	1·8169	1·550	·645
·356	2·808	·5527	·22926	1·8089	1·552	·644
·357	2·801	·5552	·22955	1·8011	1·555	·643
·358	2·793	·5576	·22983	1·7932	1·557	·642
·359	2·785	·5600	·23011	1·7855	1·560	·641
·360	2·777	·5625	·23040	1·7777	1·562	·640
·361	2·770	·5649	·23067	1·7700	1·564	·639
·362	2·762	·5673	·23095	1·7624	1·567	·638
·363	2·754	·5698	·23123	1·7548	1·569	·637
·364	2·747	·5723	·23150	1·7472	1·572	·636
·365	2·739	·5748	·23177	1·7397	1·574	·635
·366	2·732	·5772	·23204	1·7322	1·577	·634
·367	2·724	·5797	·23231	1·7247	1·579	·633
·368	2·717	·5822	·23257	1·7173	1·582	·632
·369	2·710	·5847	·23283	1·7100	1·584	·631
·370	2·702	·5873	·23310	1·7027	1·587	·630
·371	2·695	·5898	·23335	1·6954	1·589	·629
·372	2·688	·5923	·23361	1·6881	1·592	·628
·373	2·680	·5948	·23387	1·6809	1·594	·627
·374	2·673	·5974	·23412	1·6737	1·597	·626
·375	2·666	·6000	·23437	1·6666	1·600	·625
·376	2·659	·6025	·23462	1·6595	1·602	·624
·377	2·652	·6051	·23487	1·6525	1·605	·623
·378	2·645	·6077	·23511	1·6455	1·607	·622
·379	2·638	·6103	·23535	1·6385	1·610	·621
·380	2·631	·6129	·23560	1·6315	1·612	·620
·381	2·624	·6155	·23583	1·6246	1·615	·619
·382	2·617	·6181	·23607	1·6178	1·618	·618
·383	2·610	·6207	·23631	1·6109	1·620	·617
·384	2·604	·6233	·23654	1·6041	1·623	·616
·385	2·597	·6260	·23677	1·5974	1·626	·615
·386	2·590	·6286	·23700	1·5906	1·628	·614
·387	2·583	·6313	·23723	1·5839	1·631	·613
·388	2·577	·6339	·23745	1·5773	1·633	·612
·389	2·570	·6366	·23767	1·5706	1·636	·611
·390	2·564	·6393	·23790	1·5641	1·639	·610
·391	2·557	·6420	·23811	1·5575	1·642	·609
·392	2·551	·6447	·23833	1·5510	1·644	·608
·393	2·544	·6474	·23855	1·5445	1·647	·607
·394	2·538	·6501	·23876	1·5380	1·650	·606
·395	2·531	·6528	·23897	1·5316	1·652	·605
·396	2·525	·6556	·23918	1·5252	1·655	·604
·397	2·518	·6583	·23939	1·5188	1·658	·603
·398	2·512	·6611	·23959	1·5125	1·661	·602
·399	2·506	·6638	·23979	1·5062	1·663	·601
·400	2·500	·6666	·24000	1·5000	1·666	·600
·401	2·493	·6694	·24019	1·4937	1·669	·599
·402	2·487	·6722	·24039	1·4875	1·672	·598
·403	2·481	·6750	·24059	1·4813	1·675	·597
·404	2·475	·6778	·24078	1·4752	1·677	·596
·405	2·469	·6806	·24097	1·4691	1·680	·595
·406	2·463	·6835	·24116	1·4630	1·683	·594
·407	2·457	·6863	·24135	1·4570	1·686	·593
·408	2·450	·6891	·24153	1·4509	1·689	·592
·409	2·444	·6920	·24171	1·4449	1·692	·591
·410	2·439	·6949	·24190	1·4390	1·694	·590
·411	2·433	·6977	·24207	1·4330	1·697	·589
·412	2·427	·7006	·24225	1·4271	1·700	·588
·413	2·421	·7035	·24243	1·4213	1·703	·587
·414	2·415	·7064	·24260	1·4154	1·706	·586
·415	2·409	·7094	·24277	1·4096	1·709	·585
·416	2·403	·7123	·24294	1·4038	1·712	·584
·417	2·398	·7152	·24311	1·3980	1·715	·583
·418	2·392	·7182	·24327	1·3923	1·718	·582
·419	2·386	·7211	·24343	1·3866	1·721	·581
·420	2·380	·7241	·24360	1·3809	1·724	·580
α	$\frac{1}{\alpha}$	$\frac{\alpha}{1-\alpha}$	$\alpha(1-\alpha)$	$\frac{1-\alpha}{\alpha}$	$\frac{1}{1-\alpha}$	$1-\alpha$

$1-\alpha$	$\frac{1}{1-\alpha}$	$\frac{1-\alpha}{\alpha}$	$\alpha(1-\alpha)$	$\frac{\alpha}{1-\alpha}$	$\frac{1}{\alpha}$	α
·421	2·375	·7271	·24375	1·3752	1·727	·579
·422	2·369	·7301	·24391	1·3696	1·730	·578
·423	2·364	·7331	·24407	1·3640	1·733	·577
·424	2·358	·7361	·24422	1·3584	1·736	·576
·425	2·352	·7391	·24437	1·3529	1·739	·575
·426	2·347	·7421	·24452	1·3474	1·742	·574
·427	2·341	·7452	·24467	1·3419	1·745	·573
·428	2·336	·7482	·24481	1·3364	1·748	·572
·429	2·331	·7513	·24495	1·3310	1·751	·571
·430	2·325	·7543	·24510	1·3255	1·754	·570
·431	2·320	·7574	·24523	1·3201	1·757	·569
·432	2·314	·7605	·24537	1·3148	1·760	·568
·433	2·309	·7636	·24551	1·3094	1·763	·567
·434	2·304	·7667	·24564	1·3041	1·766	·566
·435	2·298	·7699	·24577	1·2988	1·769	·565
·436	2·293	·7730	·24590	1·2935	1·773	·564
·437	2·288	·7761	·24603	1·2883	1·776	·563
·438	2·283	·7793	·24615	1·2831	1·779	·562
·439	2·277	·7825	·24627	1·2779	1·782	·561
·440	2·272	·7857	·24640	1·2727	1·785	·560
·441	2·267	·7889	·24651	1·2675	1·788	·559
·442	2·262	·7921	·24663	1·2624	1·792	·558
·443	2·257	·7953	·24675	1·2573	1·795	·557
·444	2·252	·7985	·24686	1·2522	1·798	·556
·455	2·247	·8018	·25697	1·2471	1·801	·555
·446	2·242	·8050	·24708	1·2421	1·805	·554
·447	2·237	·8083	·24719	1·2371	1·808	·553
·448	2·232	·8115	·24729	1·2321	1·811	·552
·449	2·227	·8148	·24739	1·2271	1·814	·551
·450	2·222	·8181	·24750	1·2222	1·818	·550
·451	2·217	·8214	·24759	1·2172	1·821	·549
·452	2·212	·8248	·24769	1·2123	1·824	·548
·453	2·207	·8281	·24779	1·2075	1·828	·547
·454	2·202	·8315	·24788	1·2026	1·831	·546
·455	2·197	·8348	·24797	1·1978	1·834	·545
·456	2·192	·8382	·24806	1·1929	1·838	·544
·457	2·188	·8416	·24815	1·1881	1·841	·543
·458	2·183	·8450	·24823	1·1834	1·845	·542
·459	2·178	·8484	·24831	1·1786	1·848	·541
·460	2·173	·8518	·24840	1·1739	1·851	·540
·461	2·169	·8552	·24847	1·1691	1·855	·539
·462	2·164	·8587	·24855	1·1645	1·858	·538
·463	2·159	·8621	·24863	1·1598	1·862	·537
·464	2·155	·8656	·24870	1·1551	1·865	·536
·465	2·150	·8691	·24877	1·1505	1·869	·535
·466	2·145	·8726	·24884	1·1459	1·872	·534
·467	2·141	·8761	·24891	1·1413	1·876	·533
·468	2·136	·8796	·24897	1·1367	1·879	·532
·469	2·132	·8832	·24903	1·1321	1·883	·531
·470	2·127	·8867	·24910	1·1276	1·886	·530
·471	2·123	·8903	·24915	1·1231	1·890	·529
·472	2·118	·8939	·24921	1·1186	1·893	·528
·473	2·114	·8975	·24927	1·1141	1·897	·527
·474	2·109	·9011	·24932	1·1097	1·901	·526
·475	2·105	·9047	·24937	1·1052	1·904	·525
·476	2·100	·9083	·24942	1·1008	1·908	·524
·477	2·096	·9120	·24947	1·0964	1·912	·523
·478	2·092	·9157	·24951	1·0920	1·915	·522
·479	2·087	·9193	·24955	1·0876	1·919	·521
·480	2·083	·9230	·24960	1·0833	1·923	·520
·481	2·079	·9267	·24963	1·0790	1·926	·519
·482	2·074	·9305	·24967	1·0746	1·930	·518
·483	2·070	·9342	·24971	1·0703	1·934	·517
·484	2·066	·9379	·24974	1·0661	1·937	·516
·485	2·061	·9417	·24977	1·0618	1·941	·515
·486	2·057	·9455	·24980	1·0576	1·945	·514
·487	2·053	·9493	·24983	1·0533	1·949	·513
·488	2·049	·9531	·24985	1·0491	1·953	·512
·489	2·044	·9569	·24987	1·0449	1·956	·511
·490	2·040	·9607	·24990	1·0408	1·960	·510
α	$\frac{1}{\alpha}$	$\frac{\alpha}{1-\alpha}$	$\alpha(1-\alpha)$	$\frac{1-\alpha}{\alpha}$	$\frac{1}{1-\alpha}$	$1-\alpha$

$1-\alpha$	$\frac{1}{1-\alpha}$	$\frac{1-\alpha}{\alpha}$	$\alpha(1-\alpha)$	$\frac{\alpha}{1-\alpha}$	$\frac{1}{\alpha}$	α
·491	2·036	·9646	·24991	1·0366	1·964	·509
·492	2·032	·9685	·24993	1·0325	1·968	·508
·493	2·028	·9723	·24995	1·0283	1·972	·507
·494	2·024	·9762	·24996	1·0242	1·976	·506
·495	2·020	·9801	·24997	1·0202	1·980	·505
·496	2·016	·9841	·24998	1·0161	1·984	·504
·497	2·012	·9880	·24999	1·0120	1·988	·503
·498	2·008	·9920	·24999	1·0080	1·992	·502
·499	2·004	·9960	·24999	1·0040	1·996	·501
·500	2·000	1·0000	·25000	1·0000	2·000	·500
α	$\frac{1}{\alpha}$	$\frac{\alpha}{1-\alpha}$	$\alpha(1-\alpha)$	$\frac{1-\alpha}{\alpha}$	$\frac{1}{1-\alpha}$	$1-\alpha$

EFFECTS OF MORPHINE SULPHATE ON CAPILLARY FILTRATION AND THORACIC DUCT LYMPH FORMATION IN THE RAT[1]

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(*Accepted for publication 13th November, 1961.*)

SUMMARY.

Rats with chronic experimental fistulae of the thoracic duct were maintained post-operatively on regimes of saline infusion given either intravenously or intragastrically. A narcotizing dose of morphine sulphate reduced lymph flow during intravenous infusion but not during intragastric infusion. This could be interpreted as due to reduction of capillary filtration rather than reduction of fluid absorption from the gut or diminished lymph propulsion. Morphia initiated a moderate hypotension of at least seven hours' duration. This hypotension was sustained well beyond the time during which lymph flow was reduced. Portal pressure and flow appeared to be unaffected by morphia. The reduced filtration following morphia has been tentatively attributed to a reduction in filtering area of the abdominal capillary bed rather than to lowered capillary filtering pressure.

INTRODUCTION.

In the early literature morphia was said to increase lymph formation in dogs (Rogowicz, 1885; Tzchirwinsky, 1894; and Vinci, 1908) although this opinion was based on inadequately controlled experiments. More recently, a prolonged decrease in the flow of thoracic duct lymph in unanaesthetized rats has been reported to occur after morphia administration (Simmonds, 1957). The effect was not apparently related to the action of morphia on gastric motility. The decrease in thoracic duct flow with morphia was confirmed in the present investigation and experiments have been carried out to clarify the mechanism of the decrease. In the first place the effect of morphia on lymph flow and haemodynamics was determined when capillary filtration and lymph flow had been deliberately increased by intravenous infusion of saline. Secondly, morphia was given during a similar increase in lymph flow produced by intragastric infusion of saline. The latter procedure should accentuate any effects of morphia on intestinal absorption of fluid or on propulsion of lymph. Since evidence sug-

[1] This work was carried out with the aid of a grant from the National Health and Medical Research Council, Canberra.

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gested that morphia decreased capillary filtration the haemodynamic effects were studied by measurement of arterial blood pressure in unanaesthetized rats and of portal venous pressure and blood flow in lightly anaesthetized rats.

Methods.

Animals. Male laboratory rats (*R. norvegicus*, hooded strain) were used for most experiments. These rats ranged in weight from 180 to 220 gm. but most commonly were 200 gm.

Anaesthesia. All experiments required surgical anaesthesia at some stage. In experiments to measure portal blood pressure and flow the animals were kept anaesthetized until they were sacrificed. Open ether was used in earlier experiments in which the rats were prepared with thoracic duct fistulae for chronic experiments. For later acute experiments in which portal pressures and flows were measured ether was found to be less satisfactory than either ethanol (5 ml. of 15 p.c. ethanol blood pressure per 100 gm. of rat, by gavage) or paraldehyde (0·1 ml. per 100 gm. of rat, intramuscularly).

Preparation and maintenance of rats with a thoracic duct fistula. This preparation was the standard one described by Bollman, Cain and Grindlay (1948). The stomach and right jugular vein were also cannulated as previously described (Simmonds, 1957). In some animals a further cannula was introduced in the left common carotid artery. This cannula was brought out through the skin of the neck with the intravenous cannula.

After operation the rats were permitted to recover from anaesthesia in a restraint cage developed from the original Bollman cage. This cage was mounted on a two-pan sliding weight balance and the whole placed in a warm-box which also carried pumps, taps, switches and a heating bulb.

When the operated rats had regained consciousness an intragastric infusion of Ringer-Locke saline at 0·6 ml. per hour was commenced. In some experiments this was continued for 16 to 20 hours until the commencement of an experimental period when infusion was increased to 2·6 ml. per hour for 7 hours and given either intragastrically or intravenously. Overnight infusion at 0·6 ml. per hour intragastrically was then resumed. In other experiments the low intragastric infusion was continued throughout the first post-operative day until 6.0 p.m. at which time infusion was increased to 3·0 ml. per hour and maintained at that rate thenceforward. No food was given throughout the three or four-day post-operative period.

Arterial blood pressure. This was measured directly *via* a composite cannula of "Transflex" with a fine nylon tip inserted into the common carotid artery. Recording was by means either of a double membrane mechanical recording manometer or by a Sanborn electromanometer in conjunction with a Both main amplifier and pen oscillograph. Both types were repeatedly calibrated during experiments with a mercury manometer.

In unanaesthetized rats in which arterial pressure was measured during the course of four days the cannula was kept patent by a continuous intra-arterial positive pressure drip of 5 IU per ml. heparin in Ringer-Locke saline at 0·2 ml. per hour from a roller pump fed by a burette. In addition, the rat was given 0·1 ml. of heparin 1000 IU per ml.) intravenously each morning. In acute experiments animals were given 0·1 ml. of heparin (1000 IU per ml.) at the end of operation prior to commencement of recording. Occasional damping of the record occurred from small clots at the cannula tip which could usually be cleared by forceful injection of a small volume of saline. There was no evidence of embolic phenomena.

Mean portal pressure. A nylon cannula with a sharp bevel was thrust directly through the wall of the exposed portal vein as near as possible to the liver. The cannula was attached to a single tube saline manometer of small bore. Care was taken at operation to minimize bleeding from the laparotomy wound and handling of the viscera. The animal was heparinized and the wound partially closed with Michel clips. The rat was kept on a warm operating table throughout.

Mean blood flow in an extracorporeal portal-to-jugular shunt. Flow was measured by the temporary diversion of blood flow from the shunt to displace a bubble along a calibrated outflow tube. After timing a known volume (0·2 ml.) the extravasated blood was returned to the shunt. It was sometimes necessary in cannulating the portal vein to tie off one or more of the gastric, splenic or mesenteric veins. Apparently anastomatic drainage was adequate since congestion of the spleen and other viscera was never evident. Rectal temperature was also measured but changes were found not to correlate with changes in portal blood flow.

Plasma protein determination in blood and lymph. Plasma protein was determined by the method of Gornall, Bardawill and David (1949) based on the biuret reaction. The method was modified for the determination of lymph protein. Epecially in more concentrated lymph, marked but variable turbidity developed. This turbidity was presumably due to lipid. The following procedure reduced turbidity and improved reproducibility.

"Analar" ether was extracted several times with the alkaline blank reagent until no further brown colour developed. Two ml. of pretreated ether were added to 5 ml. each of the reaction mixture and the blank. The two liquids were agitated together by stirring and then separated by centrifugation. The overlying ether layer was removed by suction and the optical densities of both reaction mixture and blank were read at 555 mμ in a Unicam S.P. 600 spectrophotometer.

In very dilute lymphs, such as those in experiments with continuous high rate infusions, this extraction was not necessary.

Morphia regime. The dose of morphine sulphate selected was 5 mg. per 100 gm. of rat. At this dose the drug induced a deep narcosis which lasted for two to three hours. Injection was always made into the jugular cannula and the dose was given in four equal portions at five-minute intervals over 15 minutes, each injection washed in by 0·1 ml. of saline. The level of narcosis was assessed by four independent "clinical" signs. These were: breathing rate, tail arching, response to noise and response to ear-tweaking. The three latter were scored on an arbitrary scale of four degrees of intensity of response. In a series of nine animals it was found that the maximum effect of morphia on these responses was reached in about twelve minutes. After thirty minutes there was a slow return to normal levels by about 150 minutes. There was very little variation between individual rats in these responses during the depth of narcosis and only a little more variation during recovery from narcosis.

RESULTS.

Effect of morphia on lymph production during repeated intravenous infusions.

The averaged results in Fig. 1 show that morphia decreased lymph flow and protein output for about two hours. This was consistently observed. The subsequent "overshoot" in lymph flow and protein output, compared with that of control animals after the same period of infusion, was seen in only three of the six rats given morphia and its significance cannot be assessed at present.

Effect of morphia on lymph production during continuous intravenous or intragastric infusions.

As Fig. 2 shows, morphia depressed lymph flow when this had been enhanced by prolonged intravenous infusion of saline, but had little or no effect on the high lymph flow produced by the same rate of infusion given intragas-

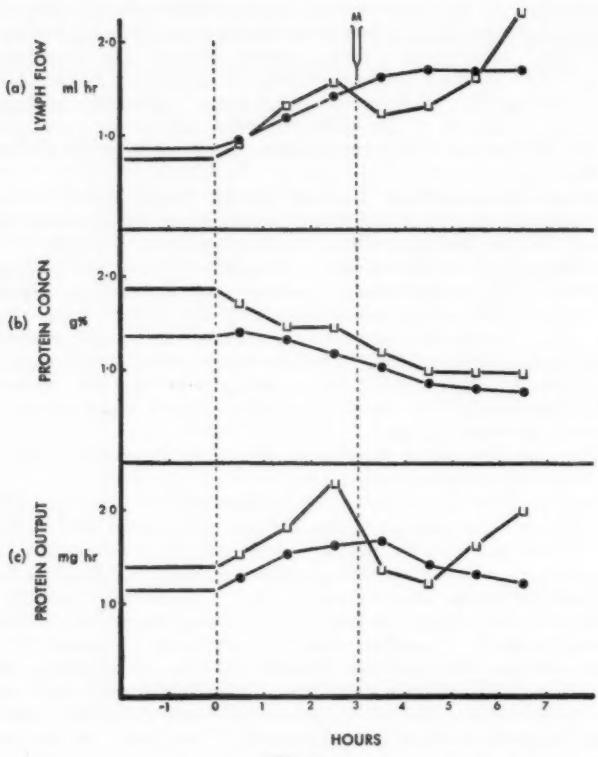

Fig. 1.

(a) Average lymph flow during increased intravenous infusion of Ringer-Locke solution at 2·6 ml./hr. starting at 0 hours.
(b) Average lymph protein concentration (same experiments).
(c) Average lymph protein output (same experiments).
⊔ == ⊔ M. Morphia given – 5 experiments.
○ —— ○ No morphia given – 7 experiments.
M = start of morphine administration.

trically. The depression of lymph flow during intravenous infusion was accompanied by a small but consistent increase in protein concentration of the lymph. Interpretation of these results was not complicated by the changing flow and protein concentration which were seen when infusion was commenced only a few hours before injection of morphia (Fig. 1).

Effect of morphia on carotid arterial blood pressure in unanaesthetized rats.

Morphia depressed arterial blood pressure by 10-20 mm. Hg. and the effect persisted for many hours (Fig. 3). This was surprising, but artefacts such as

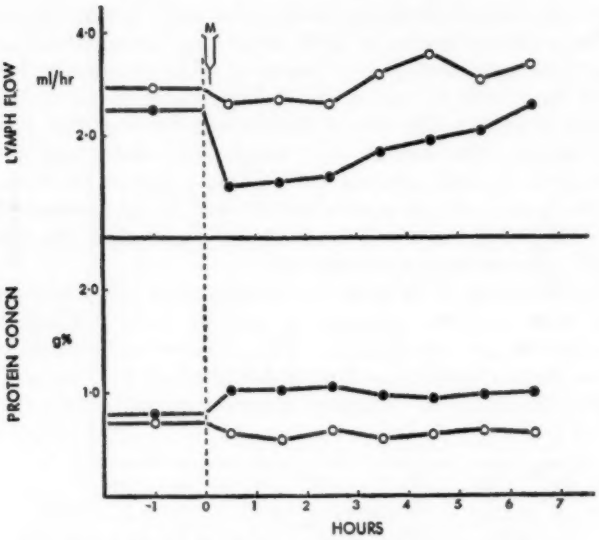

Fig. 2. Effect of morphia on lymph flow and protein concentration during continuous infusion of Ringer-Locke solution at 3·0 ml./hr.; given either intravenously or intragastrically.

○ == ○ Intragastric infusion – 4 experiments.
● == ● Intravenous infusion – 6 experiments.
M = start of morphine administration.

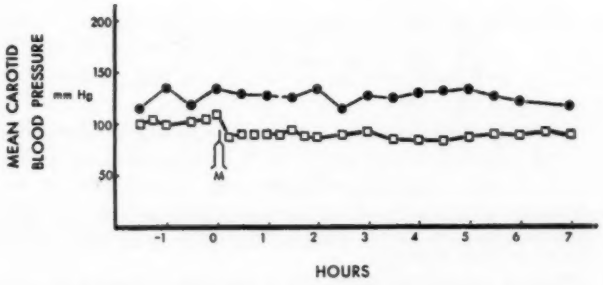

Fig. 3. Effect of morphia on carotid blood pressure in unanaesthetized rats with thoracic duct fistula, during continuous increased infusion (3·2 ml./hr.) of Ringer-Locke solution on second and third post-operative days.

● —— ● No morphia – 4 experiments.
□ == □ Morphia – 4 experiments.
M = start of morphine administration.

coincidence with diurnal fluctuation seem to be ruled out by the control observations. These measurements, in both control and morphinized animals were made during prolonged intravenous infusion of saline in rats with lymph fistulae. Fig. 4 shows the results in another set of experiments in which blood pressure and heart rate were recorded almost continuously for one hour before and one hour after morphia. The thoracic duct was not cannulated and no intravenous infusion was given in these animals. There was a decrease in heart rate (Figs. 4 a and b) and a drop in both systolic and diastolic blood pressure after morphia (Fig. 4c). The diastolic blood pressure fell more than the systolic blood pressure, and pulse pressure was increased.

A transient slowing of the heart and depression of blood pressure were seen immediately after the first injection of morphia (see Methods for morphia regime) in four of five experiments. This transient response is to be distinguished from the sustained fall which is described above. As noted by Evans *et. al* (1952) the transient depressor response exhibited tachyphylaxis, with

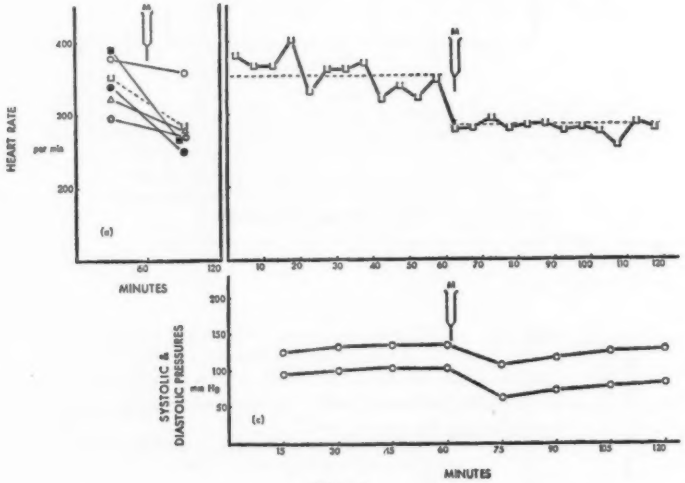

Fig. 4.

(a) Effect of morphia on heart rate in unanaesthetized rats (not infused). Individual animals. Each point is the average of all mean heart rates before or after morphia.

(b) Effects of morphia on heart rate in the above experiments; average heart rates at five-minute intervals before and after morphia.
M = start of morphine administration.

(c) Effect of morphia on systolic and diastolic pressures in four unanaesthetized non-infused rats at 15-minute intervals before and after morphine administration.
○ == ○ Average systolic pressure.
● == ● Average diastolic pressure.

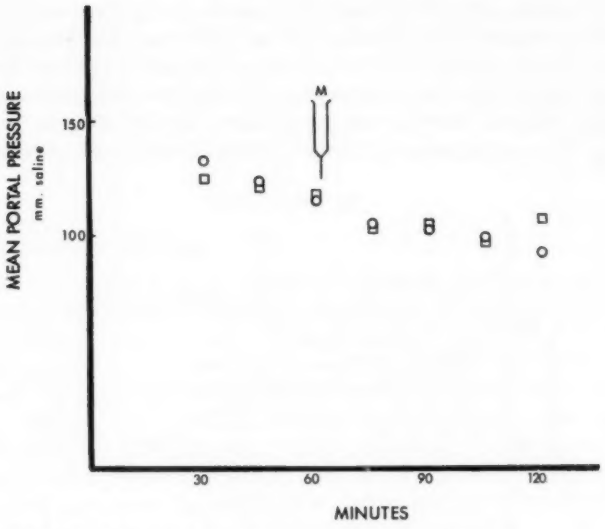

Fig. 5. Effect of morphia on portal venous pressure.
○ —— Controls – no morphia.
□ ——Morphia – 5 mg./100 g. rat.
M = start of morphine administration.

little or no effect observed after injection of 2nd, 3rd or 4th portions of the divided dose of morphia.

Effect of morphia on portal venous pressure in anaesthetized rats.

Fig. 5 shows two groups of experiments in which mean portal pressure was measured for two hours after laparotomy and cannulation. In the first group of eight rats morphia was given one hour after laparotomy. In the second group of twelve rats no morphia was given. Although mean portal pressures fell continuously in both groups throughout the two-hour period no effect could be attributed to morphia. The fall in portal pressure could, however, be attributed to general circulatory depression presumably due to anaesthesia and operation. Thus in five of the twelve control experiments arterial pressure was also measured and found to fall to between 70-80 mm. Hg. during the second hour.

Effect of morphia on portal venous blood flow in anaesthetized rats.

As with portal pressure measurement technical limitations necessitated anaesthesia for the measurement of portal blood flow. This had the concomitant limitation of general circulatory depression. Thus in four experiments (without

morphia) in which arterial pressure was measured together with portal blood flow, arterial pressures were found to fall to 60-70 mm. Hg. during the second hour of experiment. Portal flow fell continuously during the two hours. However, this degree of circulatory depression was not considered to preclude tests for a possible effect of morphia on portal flow. In Fig. 6 mean portal flow in three groups of experiments is presented: a control group received no morphia,

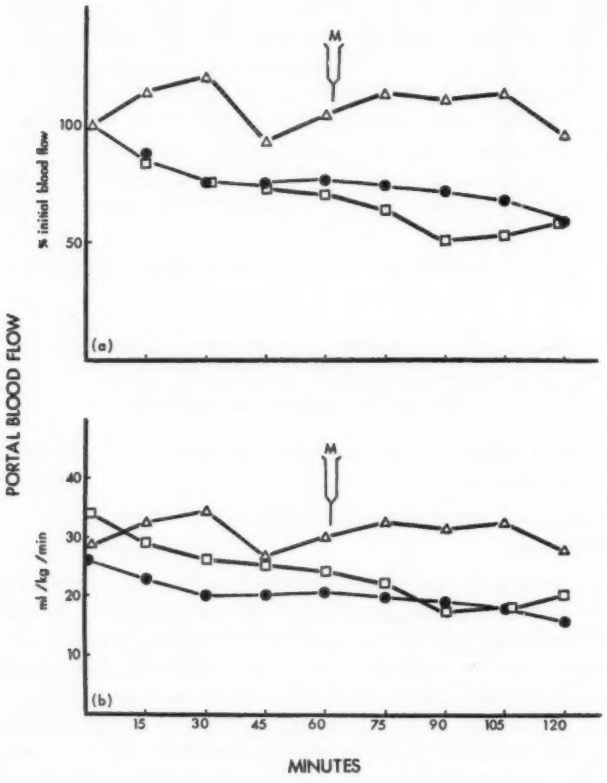

Fig. 6. Effect of morphia on mean portal blood flow.
(a) Blood flow as a percentage of the immediate post-operative flow rate.
(b) Blood flows expressed as ml./kg./min.
● —— ● Controls: ether anaesthesia, replacement transfusion, no morphia.
□ == □ Post-operative morphia only, 5 mg. per 100 g. rat.
△ == △ Pre-operative morphia, 5 mg. per 100 g. rat I.P. 15 minutes before operation and post-operative morphia as above.

one of the experimental groups was given morphia 60 mins. after the first measurement of portal blood flow and the other received, in addition, a pre-operative dose of morphia. Portal blood flow was somewhat depressed by morphia given only post-operatively but no such effect was seen when the post-operative dose followed about one and a half hours after a pre-operative injection. The interpretation of these results will be considered later.

DISCUSSION.

Morphia and capillary filtration.

Intestinal tissue fluid may be expanded either by increased capillary filtration or by rapid absorption of fluid from the lumen of the gut. In either case an increased flow of lymph occurs. Morphia depressed lymph flow only when this had been increased by intravenous infusion and had no effect when flow was increased to a similar extent by intragastric infusion of saline. These results (Figs. 1 and 2) provide good evidence that morphia in some way reduces capillary filtration but has little effect on absorption of fluid from the intestinal lumen. Nor is it likely that morphia decreases propulsion of lymph, since such an effect should have been apparent when lymph flow was increased by intragastric infusion.

The mechanism of decreased capillary filtration.

No firm conclusion on this point can be drawn at present. Although Schmidt and Livingston (1933) reported that even very large doses of morphia did not lower blood pressure in the etherized rat, rabbit or guinea pig, the present experiments showed a moderate but sustained decrease in arterial pressure in the unanaesthetized rat. The mechanism of this hypotensive effect is not clear. To some extent, it may have been due to the reduction of physical activity during narcosis which would account for the discrepancy between these results and those of Schmidt and Livingston (1933) from anaesthetized rats. However, no clear correlation was observed clinically and, as noted, the hypotensive effect outlasted the more obvious narcotic effects of the morphia. It was also significant that the decrease in blood pressure outlasted the depression of lymph flow in unanaesthetized rats with lymph fistulae. It did not appear, therefore, that the decrease in capillary filtration could be ascribed simply to a decrease in filtration pressure associated with lowered arterial blood pressure.

Although inconclusive, experiments on splanchnic haemodynamics did not point to an arteriolar vasoconstriction in this region which might have lowered capillary pressure more than would be suggested by arterial pressure measurements. The experiments were inconclusive because it was apparent that anaesthesia and operation had, in themselves, a depressant effect. However, this did not seem to be severe enough to mask a possible vasoconstrictor effect of morphia. Thus, portal venous pressures lay between 90 and 115 mm. saline during the second hour of observation. This compares with the range of 80-120 mm. given

by Reynell (1952) and a "normal" portal pressure of 14·1 ± 1·4 cm. reported by Lake (1955). Portal venous blood flows during the second hour of observation were greater than 20 ml./kg./min. This compares favourably with a flow of about 30 ml./kg./min. which can be calculated by combining the data of Sapirstein (1956) and Bullard (1959). The values would be at the lower end of the range for "normal" portal blood flows estimated from bromosulphthalein clearances in the dog (e.g. Smythe and Gilmore, 1955). Portal venous pressures and flows, then, lay within the normal range although arterial blood pressure was lower than in intact unanaesthetized animals before morphia was given. After morphia the portal venous pressure – which should vary with capillary blood pressure – was unaffected. Portal blood flow was diminished but such an effect was not observed in rats premedicated with morphia. This paradoxical result may be attributable to the degree of respiratory depression which occurred when morphia was given to these animals with a portal-jugular venous shunt diverting a large part of the hepatic blood supply. The pre-operative injection of morphia allowed much shallower levels of anaesthesia to be maintained until the post-operative dose, although much of the pre-operative narcosis may have worn off before the second dose of morphia was given. Respiratory depression in such circumstances was less than when morphia was given only post-operatively to a fully etherized animal. Even in the latter case the decrease in portal blood flow was not large and did not suggest a gross splanchnic vasoconstriction. It also seemed unlikely that a flow-limited filtration could account for the reduced production of tissue fluid.

If it is accepted that the haemodynamic experiments provide evidence against a reduction of capillary blood pressure by morphia, three possibilities remain – a reduction of capillary permeability, increased colloid osmotic pressure of the blood, or a reduction in capillary filtering area due perhaps to shunting of blood past the true capillaries. A reduction in splanchnic capillary permeability seems unlikely. In fact, an increased permeability has been reported in some experiments; either as a direct action on capillaries (Krueger, Eddy and Sumwalt, 1941) or by an indirect action via histamine release (Feldberg and Paton, 1951; Evans *et al.*, 1952; Gershon and Shaw, 1957) or serotonin release (Bhattacharya and Lewis, 1956). An increase in visceral capillary permeability could not be reconciled with a reduction in lymph *and* in protein output (Fig. 2). A selective increase in capillary permeability elsewhere than in the visceral bed might reduce capillary filtration indirectly, by haemoconcentration and increase in oncotic pressure. Experiments, not reported here in detail, suggested that morphia might cause some haemoconcentration in rats. The haemoglobin in heart blood from 20 morphinized rats was 13·41 gm./100 ml. compared with 12·47 gm./100 ml. in 43 controls. The difference was statistically significant ($P < 0·01$). Plasma protein concentrations were not determined but it seemed unlikely that variation in colloid osmotic pressure could be solely responsible for the prompt and considerable decrease in lymph flow when morphia was given during intravenous infusion of saline.

There is a remaining possibility, that morphia acts on the minute blood vessels, restricting the capillary filtering area. This merits further study. Measurements were made of haemoglobin content in small segments of intestine in which the circulation had been suddenly arrested by a specially designed clamp. Results were too variable to warrant description in detail here. However, the use of isotopic techniques such as those described by Friedman (1959) might be a more fruitful approach.

Acknowledgments. I am indebted to Professor W. J. Simmonds for his valuable advice and support during this investigation. I wish to thank Miss H. Buchan for technical assistance and Mr. N. S. Stenhouse, C.S.I.R.O. Division of Mathematical Statistics, for his generosity in making statistical analyses.

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THE PRODUCTION OF GREEN PIGMENTS FROM THE BLOOD PIGMENT[1]

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(*Accepted for publication 4th October, 1961.*)

SUMMARY.

The oxidative production of green pigments from horse oxyhaemoglobin with ascorbic acid is accelerated by fluorides, ammonium chloride and by various organic nitrogenous substances such as amines, diacetyl monoxime and anthranilic acid. When cyanide and sodium dithionite replace ascorbic acid, fluorides inhibit but ammonium chloride and some nitrogenous substances accelerate green pigment formation. Diacetyl monoxime and N-phenylanthranilic acid together increase the yield of bile pigment when oxyhaemoglobin is treated with ascorbic acid.

INTRODUCTION.

The production *in vitro* of green pigments from derivatives of haemoglobin has in general advanced along three lines: the action on oxyhaemoglobin of hydrogen peroxide and sodium cyanide ("pseudohaemoglobin", Barkan and Schales, 1937); the joint action on oxyhaemoglobin of sodium dithionite, sodium cyanide and oxygen ("cruoralbin", Holden, 1943) and the effects on oxyhaemoglobin or methaemoglobin of ascorbic acid and oxygen ("choleglobin", Lemberg *et al.*, 1938). Extensive researches by Lemberg and his colleagues (Foulkes, Lemberg and Purdom, 1951) have established the close relationship between the above green pigments and have proved that the bile pigments obtained from solutions of choleglobin are derived from verdohaematin compounds present as impurities and not from choleglobin itself. It has been previously shown (Holden, 1945, 1946) that cruoralbin does not yield bile pigments and pseudohaemoglobin only traces.

From the experiments described below it was hoped to learn something about the mechanisms of the formation of choleglobin and cruoralbin. Attempts were made to alter the speed of the reactions by the addition of various substances to the reacting mixtures. A few other experiments in this field are added.

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[1] This work was aided by a grant from the National Health and Medical Research Council, Canberra.

MATERIALS AND METHODS.

Horse haemoglobin derivatives were used. For the experiments with ascorbic acid the washed red cells were laked and destromatized with toluene and CO_2; the haemoglobin was crystallized and stored at 0° C. as a paste of crystals. For the experiments with cyanide various specimens of oxyhaemoglobin were used (laked cells, cells destromatized with toluene and NaCl or with acetic acid at pH 5·6) without any quantitative or qualitative difference being observed in their behaviour. The concentration of the oxyhaemoglobin solutions used with ascorbic acid ranged from 1·5 to 2·5 p.c.; those used for the cyanide experiments from 5 to 10 p.c.

Experiments were carried out at laboratory temperature, 20-26°. The longer reaction time with ascorbic acid was compensated by less denaturation and less precipitation of insoluble pigments. The experiments with ascorbic acid were performed in volumes of 10-15 ml. of fluid in 100 ml. beakers or conical flasks. Those with dithionite and cyanide were carried out in 150 ml. conical flasks, some rotated with their axes horizontal, others upright and stationary. With the latter, less dithionite was needed but the reaction was slower. The analyses were done on a Hilger "Uvispek" spectrophotometer after treating a suitably diluted sample with CO, NaOH and $Na_2S_2O_4$ (Lemberg, 1941b). The absorption figures are arbitrary and are simply for comparison purposes.

RESULTS.

Choleglobin and cruoralbin formation.

It was found that NH_4Cl accelerated the formation, with some increase in yields, of both choleglobin and cruoralbin. The reaction was allowed to proceed without agitation for 4½ hours in a layer 0·5 cm. deep. The details are set out in Table 1.

Similar experiments with fluoride are summarised in Table 2. This substance accelerated the formation of choleglobin chiefly during the first two hours though a good deal of fluormethaemoglobin was formed. On the other hand, it strongly inhibited the formation of cruoralbin. No simple explanation of these data appears as yet.

TABLE 1.

	Choleglobin		Cruoralbin	
oxyhaemoglobin (ml.)	2·0	2·0	2·0	2·0
10 p.c. NH_4Cl (ml.)	5·0	—	3·0	—
conc. NH_4OH (ml.)	3·0	—	—	—
10 p.c. K_2CO_3 (ml.)	—	0·3	—	—
10 p.c. NaCN (ml.)	—	—	3·0	3·0
4 p.c. boric acid (ml.)	—	—	—	9·0
ascorbic acid (mg.)	10·0	10·0	—	—
$Na_2S_2O_4$ (mg.)	—	—	15	15
H_2O (ml.)	3·5	8·7	6·0	—
pH	9·0	8·8	9·4	9·3
Absorption of CO derivative of reduced pigment diluted 6 fold in NaOH				
570 mμ	1·20	1·38	0·31	0·38
625 mμ	0·80	0·48	0·66	0·44

Other types of experiment showed that fluormethaemoglobin is much more slowly reduced than methaemoglobin by both ascorbic acid and sodium dithionite. When potassium fluoride is added to methaemoglobin in 10 p.c. concentration the reduction, even by dithionite, is very slow. The difference in the behaviour of fluoride according to whether ascorbic acid or cyanide and sodium dithionite is used recalls a divergence in the behaviour towards carbon monoxide. Lemberg (1939) showed that "carbon monoxide does not prevent the formation of choleglobin from haemoglobin by hydrogen peroxide". In our experience when carbon monoxide haemoglobin is treated with sodium cyanide and hydrogen peroxide no green pigment is formed except a little on the surface of the resulting foam due to the prior conversion of a little carbon monoxide haemoglobin to oxyhaemoglobin.

Some organic compounds were tested with the following results. Ethylamine promotes the formation of choleglobin at pH 8·4 and of cruoralbin at pH 10. Dimethylamine and diethanolamine behave like ethylamine. p-Aminobenzoic acid has no effect on the formation of choleglobin at pH 8·4 or of cruoralbin at pH 10. Anthranilic acid, N-phenylanthranilic acid and diacetyl monoxime all accelerate choleglobin formation. Diacetyl monoxime only slightly accelerates the formation of cruoralbin.

Alterations in yield of bile pigments.

In four 100 ml. beakers loosely covered with watch-glasses were placed the following fluids. The volumes are in ml.

	1	2	3	4
Oxyhaemoglobin (2·2 p.c.) destromatized	10	10	10	10
Water	5	2·5	2·5	0
Diacetyl monoxime (2 p.c.) ...	0	2·5	0	2·5
N-phenylanthranilic acid (1 p.c. As the sodium salt)	0	0	2·5	2·5
Ascorbic acid (2 p.c.)	1	1	1	1

Initial pH 8·1. Incubated for 4 hr. at 26°.

To each was then added a further 1 ml. of ascorbic acid solution and 2 volumes (34 ml.) of glacial acetic acid. They were separately worked up with ether and HCl as described by Lemberg (1941b) with the following results: No. 1 yielded a little bilirubin and biliviolin; Nos. 2 and 3 yielded more biliviolin than No. 1 and in No. 4 the yield of biliviolin was much greater and little haematin remained. The biliviolin was a feebly basic one, extracted with 5 N HCl and too unstable for spectrophotometry. The decomposition of the oxy-

TABLE 2.

	Choleglobin[1]		Cruoralbin[2]	
oxyhaemoglobin (ml.)	1·0[3]	1·0	3·0	3·0
10 p.c. K_2CO_3	—	0·3	—	—
5 p.c. K_2HPO_4 (ml.)	5·0	5·0	—	—
40 p.c. w/v KF (ml.)	2·0	—	5·0	—
1·3 p.c. KCN (ml.)	—	—	3·0	3·0
ascorbic acid (mg.)	12	12	—	—
10 p.c. NH_4Cl (ml.)	—	—	3·0	3·0
$Na_2S_2O_4$ (mg.)	—	—	90[4]	90[4]
H_2O (ml.)	2·0	3·7	—	5·0
pH	8·28	8·24	9·9	9·7
Absorption after dilution as in Table 1.				
570 mμ	0·91 (0·78)	1·05 (0·88)	0·826	0·661
625 mμ	0·48 (0·71)	0·19 (0·57)	0·319	0·691

[1] the data are presented for 2 hrs. and in brackets for 4½ hours.
[2] flasks rotated until dithionite oxidised.
[3] destromatised oxyhaemoglobin.
[4] added gradually.
[5] spectrophotometry as above.

haemoglobin by ascorbic acid appears thus to be greatly accelerated and to be diverted from the choleglobin pathway to one which gives a stage of oxidation beyond that of verdoglobin but in the same general direction; verdoglobin on decomposition with acid yields biliverdin. Foulkes and Lemberg (1949) showed that the formation of choleglobin is accelerated by the presence of small amounts of alkali-treated proteins and by pyridine, mainly by diminishing the initial "lag" period.

It seems from their paper that the end-products formed in the presence of such accelerating agents were the same as in their absence. Here, however, is a "promoter" (Holden, 1947) which urges the decomposition of the blood pigment in a bile pigment direction and away from the physiologically less interesting choleglobin group. It does not seem to "perturb" any discernible amount of the blood pigment.

Some reactions of methaemoglobin.

The similar magnetic susceptibility of cyanate methaemoglobin and fluormethaemoglobin are held to indicate similar structures around the iron atom; the effect of potassium cyanate on the reduction of methaemoglobin was therefore studied. Unlike potassium thiocyanate, potassium cyanate does not denature blood pigment derivatives. A solution of methaemoglobin or of oxyhaemoglobin may be saturated with potassium cyanate without either protein being denatured. potassium cyanate retards the reduction in neutral solution of cyanate methaemoglobin even more than potassium fluoride retards the reduction of fluormethaemoglobin in neutral solution. Unlike fluoride, however, potassium cyanate retarded the formation of choleglobin. Sodium formate, which is said to combine with methaemoglobin, had no effect on the formation of cruoralbin.

The effects of various substances on the autoxidation of oxyhaemoglobin to cyanmethaemoglobin in the presence of sodium cyanide were examined. Ammonium chloride and ethylamine hydrochloride each accelerated the oxidative conversion of oxyhaemoglobin to cyanmethaemoglobin. If the fluid be less alkaline than pH 10 the conversion in the presence of 3 p.c. sodium cyanide is not very fast. If about 3 p.c. of ammonium chloride was added, instead of only 10 p.c. of the oxyhaemoglobin being converted to cyanmethaemoglobin in 4 hours about 95 p.c. was converted. A striking experiment was performed by taking 1 ml. of strong oxyhaemoglobin solution and adding 1 ml. of 10 p.c. sodium cyanide. After adding two drops of concentrated ammonia the pigment was changed to cyanmethaemoglobin in 20 minutes. Although the solution was as alkaline as pH 11·3 a sample on dilution and reduction showed the haemochromogen-like spectrum usually seen on reducing cyanmethaemoglobin. This spectrum rapidly changed to that of haemoglobin showing that no denaturation had occurred.

Tsushima (1954) mentions a similar transient haemochromogen spectrum when methaemoglobin is reduced with sodium dithionite in the presence of just enough sodium benzoate to convert the methaemoglobin to parahaematin but without denaturing the protein. The benzoate concentration is insufficient to prevent the initially formed haemochromogen from changing to haemoglobin. A similar effect, less well understood, can be produced as follows: To 2 ml. of 2 p.c. methaemoglobin are added 0·05 ml. of 10 p.c. potassium carbonate and 0·8 ml. of 0·1 M formaldehyde. In spite of the solution being at pH 10 a good deal of (apparently) acid methaemoglobin is formed. A drop of concentrated ammonia is added and immediately a few crystals of sodium dithionite. The spectrum is then that of haemochromogen which within a few seconds mostly changes to that of haemoglobin. If about half a minute elapses between the addition of ammonia and that of the dithionite little haemochromogen is seen. The presence of the ammonia appears to be essential. If sodium sulphite is used to remove the formaldehyde, or if (Holden, 1947) the dithionite is added directly to the mixture containing free formaldehyde, little haemochromogen is seen. If more formaldehyde is used, as in some of Tsushima's (1956) experiments, most of the methaemoglobin is denatured.

The haemochromogen spectrum that is seen when haemoglobin is dried *in vacuo* or when it is precipitated with alcohol in the absence of oxygen, unlike that produced by the treatment of haemoglobin with sodium benzoate or sodium salicylate, corresponds only to about half the haem present. It is as yet uncertain whether the other half of haem is seen as such or remains as haemoglobin despite the removal of water. Whatever be its temporary condition, it can all be restored to haemoglobin. If methaemoglobin is dried at about pH 6 all the pigment is temporarily converted to the parahaematin form and is restored to methaemoglobin on the addition of water.

DISCUSSION.

Experiments on the formation of green pigments from the blood pigment show how far are most of them from providing a picture of the complete conversion of oxyhaemoglobin to bile pigment which occurs in the animal body. Foulkes, Lemberg and Purdom (1951) have disproved the contention of Lemberg *et al.* (1941b) that bile pigments are the iron-free prosthetic group of choleglobin. This paper indicates that the reaction involving the blood pigment and reducing substances may be modified, at least with regard to speed, by a variety of adjuvant substances.

The current view of the blood pigment envisages that the haem or haematin molecule lies between and is united to a pair of iminazole molecules appropriately situated on the protein. The experiments of this paper suggest that other modes of attachment to the protein both of the pigment and of other molecules should receive consideration.

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GLUCOSE METABOLISM IN HUMAN (THIERSCH) SKIN GRAFTS

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(*Received for publication 8th November, 1961.*)

SUMMARY.

Glucose metabolism in human split skin (Thiersch) grafts, when measured *in vitro*, is shown to be mainly by glycolysis with a low rate of oxygen uptake. Doubling of glucose uptake and lactate production as well as oxygen uptake by 0·1 mM 2:4-dinitrophenol (DNP) is interpreted as supporting the conception that this glycolytic activity is not simply due to inadequate oxygen perfusion but is a feature of skin metabolism *in vivo*. The ability of skin to maintain an oxygen uptake for at least four hours without substrate suggested that skin glycogen might be the endogenous carbohydrate capable of sustaining respiration but measurements of glycogen levels in skin before and after incubation demonstrate that glycogen itself is unlikely to provide this pool, at least in the *in vitro* system.

INTRODUCTION.

The importance of studying the metabolic processes in skin stems from three considerations. Firstly, this structure may be obtained as a composite cellular mass in a relatively organised state which can be studied *in vitro* under conditions which approximate relatively closely to those conditions *in vivo* in which its survival and growth are known to occur unhampered. The conditions under which free grafts of skin survive, at least during the first 48 hours of the grafting procedure, are those of plasmatic perfusion (Conway *et al.*, 1951) and this can be reproduced *in vitro* by utilizing the conventional Warburg manometer. Secondly, skin must have a robust metabolic system to function efficiently through a wide temperature range which in humans extends from 25° C. to 40° C. Thirdly, it retains its ability to grow and divide after transplantation, even after prolonged storage at low temperatures and under unfavourable conditions (Billingham and Medawar, 1952). Thus skin offers a unique opportunity to examine the integration of metabolic processes in a living mammalian tissue still organised in structure and under conditions which allow growth and cell division. Since a considerable amount of histochemical evidence (Lobitz and Holyoake, 1954; Scothorne and Scothorne, 1953) already exists about glycogen deposition and utilization in skin following injury a study of carbohydrate metabolism in human Thiersch grafts was undertaken.

METHOD.

For these experiments medium thickness Thiersch graft was taken from the thighs of healthy human donors between ages of 5 years and 60 years. The material was stored at about 4° C. until it was used. The skin was cut into squares about 10 sq. mm. in area and about 3 sq. cm. used for each Warburg vessel. Larger quantities were used for studies on glycogen. The skin in each vessel was weighed after the metabolic studies were completed.

The manometric studies were carried out in a conventional Warburg apparatus. Each vessel contained 3 ml. of Hank's salt solution at pH 7·4 with 50 μg. of streptomycin/ml. and, where appropriate, the following: glucose 0·056 M; 2:4-dinitrophenol (DNP) 0·1 mM; sodium iodoacetate 0·01 M; sodium DL-lactate 6 mM; sodium pyruvate 7 mM. For measurements of oxygen uptake the centre well contained 0·2 ml. of 5 M KOH. Measurements were normally made over four hours. For studies of glycogen metabolism, the vessels contained 10 ml. of solution and KOH was omitted from the centre well.

Glucose was determined by the method of Haslewood and Strookman (1939) and lactate by the method of Barker and Summerson (1941).

Glycogen was determined as follows: The pieces of skin were shaken with 34 p.c. (w/v) of trichloroacetic acid for 20 minutes to remove glucose and soluble esters. The pieces were then transferred to graduated centrifuge tubes and heated in a boiling water bath with 5 ml. of 30 p.c. (w/v) KOH until complete solution. 5 ml. of hot ethanol were added and tubes immediately centrifuged at 2000 r.p.m. for 30 min. at 5° C. The supernatants were discarded and the precipitate washed with cold ethanol and recentrifuged at 5° C. This precipitate was digested with 2 ml. of N HCl by heating in a boiling water bath for two hours, the solution being allowed to concentrate during this period. After removal from the bath the pH was adjusted to approximately 7 with 40 p.c. (w/v) NaOH and the volume adjusted to 1·0 ml. with water. In radioactivity studies 0·1 ml. of this solution was removed for counting. The remaining 0·9 ml. was used for glucose estimation.

In radioactive solutions the final activity of substrates were: glucose-^{14}C (uniformly labelled), 1 μc./mg.; sodium lactate-1-^{14}C, 2 μc./mg.; and sodium lactate-2-^{14}C, 4 μc./mg. These chemicals were obtained from the Radiochemical Centre, Amersham, Bucks. Counting was performed with gas flow counter (Nuclear, Chicago) and end-window Geiger counter (Ekco) under standard conditions.

RESULTS.

The glucose uptake of skin measured during the incubation with 0·056 M glucose as substrate had a mean value (14 experiments) of 9·0 μmoles/gm. of wet weight/hr. with a range of values in individual experiments from 4·2 to 19·0. The mean lactate production under the same conditions was 11·2 μmoles/gm. of wet weight/hr. with a range 5·0 to 18·0, and mean oxygen uptake of 11·9 μmoles/gm. of wet weight/hr. with a range 4·5 to 19·0. Although the metabolic activity varied greatly from batch to batch, within each batch the results were in substantial agreement. When the lactate production and glucose uptake in each experiment were compared it was apparent that the majority of the glucose utilized had been converted to lactate. The mean value of the lactate produced/glucose utilized ratio was 1·25 (± 0·20 s.d.).

Table 1 shows the endogenous metabolic activity compared to that when glucose was present. This Table also shows the effect of 7 mM. pyruvate as the

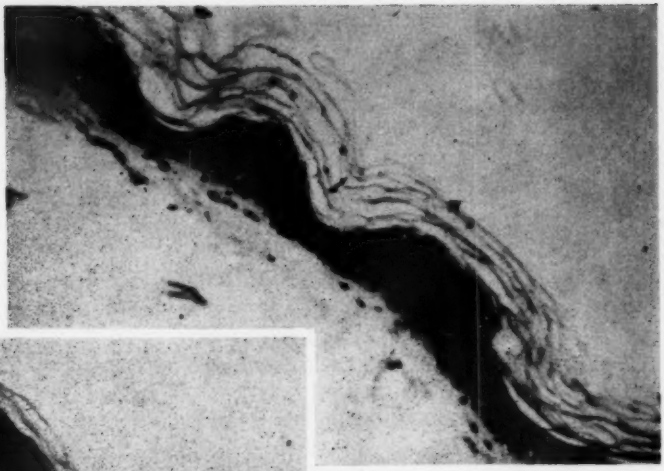

Fig. 1(a)

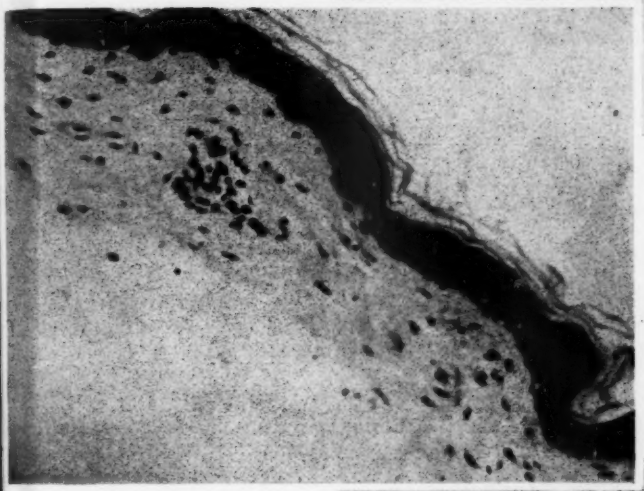

Fig. 1(b)

Fig. 1(c)

Fig. 1. This shows three sections of human skin cut as for Thiersch grafts: (a) a thin graft, (b) intermediate, (c) almost full thickness graft. This demonstrates increasing dermal elements with little increase in cellularity. (Magnification x 120.)

TABLE 1.

This shows oxygen uptake and lactate production by skin in buffered saline pH 7·4 with or without substrates. Activities are expressed as μmoles/gm. of wet weight/hr.

Experiment No.		Oxygen utilized	Lactate produced
I	Buffered saline alone	14·1	2·5
	+0·056 M glucose	14·9	11·1
II	Buffered saline alone	9·3	1·6
	+0·056 M glucose	5·7	9·3
III	Buffered saline	24·5	1·8
	+7mM pyruvate	25·0	0

TABLE 2.

The effect of 0·1 mM DNP or 0·01 M Sodium iodoacetate on metabolic activity of skin in buffered saline pH 7·4 with 0·056 M glucose as substrate. Each treated group is compared with its own control. Activities expressed as μmoles/gm. of wet weight/hr.

Experiment No.		Mean glucose uptake	Mean lactate production	Mean oxygen uptake
I	Control	8·8	9·9	5·7
	+0·1mM DNP	16·9	22·4	10·8
II	Control	19·0	11·1	14·9
	+0·1mM DNP	32·4	21·5	20·7
III	Control	10·0	10·0	15·9
	+Iodoacetate 0·01M	0	0	4·9
IV	Control	15·8	11·1	14·9
	+Iodoacetate 0·01M	0	0	4·5

TABLE 3.

This shows the effect on glycogen levels in skin of incubation at 30° C. in buffered saline pH 7·4 either alone, with 0·056 M glucose, or with 6 mM lactate. The glycogen content was estimated after 3 hours' and 8 hours' incubation and is expressed as mg./100 gm. of wet weight.

Experiment No.	Medium	Initial level	3 hours	8 hours
I	Buffered saline	12·7	7·3	5·5
	+6mM lactate	12·7	6·9	8·9
	+0·056M glucose	12·7	11·3	10·5
II	Buffered saline	15·4	8·4	11·3
	+6mM lactate	15·4	5·0	—
	+0·056M glucose	15·4	8·6	9·9
III	Buffered saline	16·8	4·7	2·4
	+6mM lactate	16·8	4·0	9·1
	+0·056M glucose	16·8	4·9	3·7
IV	Buffered saline			
	+6mM lactate	16·3	5·9	6·9
	+0·056M glucose	16·3	6·9	6·0

substrate contrasted with the endogenous metabolic activity of control skin. It will be noted that oxygen uptakes within each group are similar but no lactate production occurred during incubation with pyruvate. As shown in Table 2 the addition of 0·1 mM. DNP caused a significant increase in oxygen uptake with similar changes in glucose uptake and lactate production; and 0·01 M sodium iodoacetate, although completely inhibiting glycolysis and glucose uptake, allowed continuation of respiration at 30 p.c. of the control level.

The mean initial glycogen level in skin (8 experiments) was 19 mg./100 gm. of wet weight with a range of 13·2-37·0 and Table 3 shows the changes in these levels following incubation in Hank's solution alone, with 0·056 M glucose and with 0·006 M lactate. In most cases this level falls to about 40 p.c. of the initial figure after three hours. With saline alone, or with glucose, this fall continues very slowly so that at eight hours little additional glycogen has been lost. With lactate as substrate there was some increase in glycogen at the end of eight hours.

The incorporation of radioactivity from labelled glucose and lactate into skin glycogen is shown in Table 4. The percentage of glycogen labelled was obtained by comparing the counts/μg. of the glycogen with the activity in the standard of the appropriate substrate. It was assumed for this purpose that universally labelled glucose would lead to labelling in all carbon atoms in the glucose fragments of the glycogen and that lactate labelled in one carbon atom would lead to glucose labelled in two carbon atoms. The rate of synthesis of skin glycogen suggested from the results in Tables 3 and 4 is slow with both substrates. In addition, with lactate as substrate carbon 2 is incorporated into the glycogen more readily than carbon 1.

TABLE 4.

Incorporation of radioactivity into glycogen after incubating skin in buffered saline pH 7·4 with the appropriate substrate. The derivation of the percentage labelled figure is explained in text.

Substrates	Experiment No.	Counts/μg. substrate /100 sec.	Count μg. glycogen 100 sec.	
			Activity at 3 hours	Activity at 8 hours
Glucose-U-^{14}C	I	1600	730 (45 p.c.)	904 (56 p.c.)
	II	250	—	99 (35 p.c.)
	III	250	27 (11 p.c.)	50 (20 p.c.)
DL-lactate-1-^{14}C	I	30	3 (10 p.c.)	2·5 (8 p.c.)
	II	40	0 (0 p.c.)	0 (0 p.c.)
	III	40	1·5 (3·5 p.c.)	1·5 (3·5 p.c.)
DL-lactate-2-^{14}C	I	69	8 (11 p.c.)	40 (58 p.c.)
	II	69	17 (25 p.c.)	39 (56 p.c.)

DISCUSSION.

The metabolic rate of different batches of skin varied considerably and it was felt that the use of wet weight as the parameter of comparison contributed largely to this. The weight of graft material depends on its thickness and an examination of Fig. 1 shows that as the graft thickness increases this additional tissue consists of dermis which is much less cellular than the epidermis. A comparison of metabolic activity in epidermis and dermis shows the former is eleven times more active on a weight basis (Freinkel, 1960). Keratinization also increases weight without increasing cellularity. For exact measurement therefore, in Thiersch grafts, a parameter based on cellularity (such as estimation of nucleic acid) would probably be the most reliable. Because of the limitations imposed by the variability of the tissue, the results presented here are considered only significant in a qualitative sense. Nevertheless, the mean uptake of glucose in these experiments certainly corresponds with other similar measurements in human skin (Freinkel, 1960). The mean oxygen uptake of these experiments also correlates well with other similar measurements in human skin (Barron, 1948; Freinkel, 1960). The active glycolysis under aerobic conditions found in these experiments is also well documented (Berenblum, Chain and Healey, 1940; Barron, 1948; Cruickshank, 1956; Freinkel, 1960) and although this activity was originally thought to be a feature of metabolism in malignant cells it has become clear that this is not so, at least in the case of skin. It is pertinent, therefore, to ask whether this pattern of high aerobic glycolysis with low oxygen uptake is a true reflection of the situation *in vivo*, or is the product of the method of examination.

It was felt that this pattern might reflect inadequate perfusion with oxygen. Increasing O_2 tension from 0·2 atm. to 1 atm. during perfusion, however, is not accompanied by any marked change in pattern (Cruickshank *et al.*, 1956). When DNP was used as a metabolic stimulant a doubling of oxygen and glucose uptake occurred with little change in the lactate/glucose ratio, suggesting that limitation of oxygen supply was not responsible for this pattern of metabolism. With pyruvate as substrate there was no stimulation of oxygen uptake and no lactate production. The high lactate content of sweat adds indirect evidence that active glycolysis is an important feature of epidermal metabolism *in vivo*.

Without substrate the O_2 uptake in skin is comparable to that with glucose for at least four hours. The accumulation of lactate suggests that this in part is due to breakdown of carbohydrate. When glycolysis was abolished by 0·01 M sodium iodoacetate oxygen uptake was maintained at only 30 p.c. of the control level. Glycogen levels were examined to test the possibility that this substance supplied much of the endogenous substrate, particularly as Wohnlich (1949) had previously claimed that skin possessed considerable capacity for glycogen synthesis from lactate.

As the results presented in this paper show, no accumulation occurred and

there was a considerable fall in skin glycogen after incubation with slow synthesis in terms of incorporated radioactivity.

The breakdown of glycogen occurring was equivalent to 0·25-0·50 μmoles glucose/hour while lactate production amounted 1·5-2·0 μmoles/hour. Oxidative metabolism from these sources therefore probably contributes little to its endogenous respiratory rate. Neptune and Foreman (1959) after a study of the endogenous respiration of rat diaphragm suggested fatty acids as probably supplying this substrate.

The unequal specific activity of glycogen derived from lactate labelled in carbon 1 or carbon 2 atom supports the belief that glucose synthesis from lactate is not simply reversal of the Embden-Meyerhof pathway of breakdown.

The asymmetrical distribution of labelling in glucose derived from pyruvate has been reported by Topper and Hastings (1949) and Noble (1959). In the study of the fate of pyruvate-2-C^{14} in the Krebs cycle, Freedman and Groff (1958) noted that whereas in the fasted animal pyruvate is mainly utilized via malate, in the animal which is given glucose the greater part of the pyruvate is metabolised by decarboxylation to a 2-carbon fragment which enters the Krebs cycle. Although the experiments reported here are too incomplete to warrant more than speculation the loss of radioactivity from carbon 1 could be accounted for by conversion of lactate to pyruvate with subsequent loss of the carboxyl group as CO_2 and formation of acetyl-CoA. Glucose synthesised from Krebs cycle intermediates would therefore be more readily labelled from the carbon 2 than the carbon 1 atoms of lactate.

Acknowledgment. The authors wish to thank Miss B. Hogg for technical assistance, and Mr. D. N. Robinson for providing many of the specimens of skin. To Professor R. K. Morton the authors are especially indebted for helpful advice, and criticism in the preparation of this manuscript.

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ELECTRON MICROSCOPIC OBSERVATIONS ON THE EMIGRATION OF LEUCOCYTES

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(*Accepted for publication 2nd November, 1961.*)

SUMMARY.

Electron micrographs have been made of the capillaries and small venules in rat skin at a time following the intradermal injection of homologous serum at which active leucocytic emigration occurs. Stages in the migration of blood leucocytes are illustrated.

Polymorphs escape from small vessels by insertion of a pseudopod into the endothelium followed by amoeboid-like migration of the cell through the wall of the blood vessel. In some cases penetration occurs down intercellular junctions but in most instances passage through endothelium at other points cannot be excluded. Appreciable delay commonly occurs at either the basement membrane or peri-endothelial cell layer before eventual escape of the cell into surrounding tissues. Monocytes apparently escape by a similar process.

After passage of leucocytes through the endothelium no residual evidence of such passage is visible. Neither endothelial cells nor the intercellular substance between them differ from their normal structure in vessels in which leucocytic emigration is occurring.

INTRODUCTION.

The adherence of leucocytes to the wall of small blood vessels and their subsequent emigration into surrounding tissues is one of the basic elements of acute inflammatory response. The appearances as seen by light microscopy of both fixed and living tissues are well established (Cohnheim, 1882; Florey, 1957) but electron microscopic studies of the process have been published from only two laboratories and these accounts differ in important respects. This paper describes observations made with the electron microscope on the escape of leucocytes from the small blood vessels of rat skin induced by intradermal injection of homologous serum (Hurley and Spector, 1961).

MATERIALS AND METHODS.

Male rats, weight 50-80 gm., from an inbred Sprague Dawley strain were used.

Sterile homologous serum (0·1 ml.) was injected intradermally into the previously shaven abdominal skin. Four hours later, at a time when leucocytic emigration was known to be maximal (Hurley and Spector, 1961), the animal was anaesthetised with ether and the deep aspect of the injected area fixed in the living animal by subdermal injection of 1 p.c. buffered osmium tetroxide (Palade, 1953). A small central area of skin was excised as rapidly as

[1] Supported by a grant from the Anti-Cancer Council of Victoria.

possible with a sharp scalpel, immersed immediately in fixative, cut into pieces about 1 × 2 mm. and fixation continued at approximately 4° C. for two hours. The specimens were then dehydrated in methyl alcohol and embedded in methacrylate (85:15, butyl:methyl) in the usual way.

For control material normal skin was used and fixed and embedded in the same way.

Silver or gold sections were cut on a Farrant ultramicrotome and mounted on carbon-coated copper grids. Most sections were "stained" for half an hour with 1 p.c. uranium nitrate, but a few were examined unstained. A Siemens Elmiskop I microscope was used employing 40 or 60 Kv.

RESULTS.

Control — small vessels of normal skin.

No leucocytes are seen adhering to the endothelium, which is composed of a complete layer of cells closely joined together such as has been described in muscle capillaries (Bennett, Luft and Hampton, 1959) and mesentery (Marchesi and Florey, 1960). At intercellular junctions the plasma membranes of adjacent cells are closely applied with no electron dense material between them. The form of such junctions shows varying degrees of complexity ranging from simple overlapping, the common form, to the interdigitation of several finger-like processes. Electron dense zones at the luminal end of intercellular junctions (Bennett *et al.*, 1959) are only occasionally seen and never conspicuous.

Mitochondria and endoplasmic reticulum of the usual form are seen within endothelial cells and small vesicles, as first described by Palade (1953), are constantly present but vary considerably in number in different areas. Some vesicles in either surface of the cell communicate with the exterior by flask-shaped openings. Both capillaries and venules have a continuous ill-defined basement membrane and outside this in venules is an irregular layer of peri-endothelial cells and fine collagen fibres.

Inflamed vessels.

Endothelium. No difference from the appearances of vessels in normal skin could be established. In particular intercellular junctions are unchanged, Palade vesicles and pinocytic "flasks", or caveolae, appear to be unaltered in number, the basement membrane is of similar appearance, and no electron dense material is seen on the luminal aspect of the cells.

Polymorphonuclear leucocytes. These are readily recognized by the prominent dark homogeneous granules in their cytoplasm which also contains mitochondria and numerous large and small vesicles.

A variety of appearances representing the successive stages of emigration can be seen and the probable sequence of events is as follows: The polymorph comes to lie immediately adjacent to the endothelium, whose surface appears unaltered. Intercellular junctions may or may not be apparent in the plane of

section at the area of contact. Nothing is visible in the narrow space between the two cells. One or more small projections are often seen on the endothelial side of the polymorph and the endothelial cell surface presents minor but not corresponding irregularities of outline.

Next the polymorph sends a pseudopod, commonly of clear hyaloplasm without granules, into the substance of the endothelium. Occasionally these pseudopods are related to a cell junction but in the majority of cases junctions are not recognizable in the plane of section at the area of indentation of the endothelium. Apparently this stage of pseudopodial insertion is of brief duration as it appears relatively infrequently in our material; the majority of polymorphs lie entirely within the lumen or have moved out to a sufficient degree to cause separation of the full thickness of the endothelium. In the absence of visible attachment belts it is impossible to determine from single sections of this stage whether emigration is occurring between cells or through the cytoplasm of a single cell. In a few instances serial sections enable more detailed reconstruction of the area of emigration and in some cases it is certain that penetration is occurring through, or immediately adjacent to, a junction between endothelial cells (Figs. 4-6, 7-9).

Following insertion of a pseudopod progressive passage of the polymorph through the endothelium occurs; commonly the leading portion of the emigrating cell is arrested at either the basement membrane or peri-endothelial cell layer until all or the greater part of the leucocyte has passed beneath the endothelium. Polymorphs completely or incompletely covered by very attenuated endothelial cytoplasm are commonly seen but free attenuated endothelial processes as described by Williamson and Grisham (1961) are not present. It appears that considerable delay may occur between passage through the endothelium and escape through basement membrane or peri-endothelial layer into surrounding tissues as cells lying in the immediately subendothelial plane are commonly seen; in some cases extensive dissection along this plane is present.

Finally, a gap is found between the cells and fibres of the peri-endothelial layer and the leucocyte passes into surrounding tissues by a similar streaming process to its passage through the endothelial layer.

No gaps extending through the full thickness of the endothelium, except those occupied by leucocytes, are visible although occasional surface depressions extending deeply into endothelial cells are present. It would appear that after passage of a leucocyte any hole in the endothelium is promptly sealed off.

Monocytes.

Cells containing few granules and numerous prominent oval mitochondria, presumptively identified as monocytes, are occasionally seen at various stages of emigration. The appearances closely resemble those of polymorphs in similar positions.

EXPLANATION OF PLATES.

Key to abbreviations:

B – basement membrane
C – caveolae
E – endothelium
J – cell junction
M – mitochondrion
N – nucleus
P – platelet
PC – peri-endothelial cell
Ps – pseudopod
R – red blood cell
V – vesicle

Fig. 1. A polymorph leucocyte with numerous granules lies in close contact with endothelium. No "cement" is apparent at the area of contact or within cell junctions (J). Small pseudopods (Ps) of the polymorph do not correspond with depressions of the endothelial surface. x 22,000.

Fig. 2. A large pseudopod of a polymorph has passed through the endothelium and is separated by basement membrane (B) from portion of two peri-endothelial cells (PC). A complicated cell junction (J) lies immediately adjacent to the area of penetration. Numerous vesicles (V) are present in endothelial cells. x 17,000.

Fig. 3. A leucocyte of indeterminate type is passing through endothelium immediately adjacent to an intact cell junction (J). Penetration reaches as far as basement membrane (B). A long tongue of endothelial cytoplasm (X) lies against the leucocyte.

Figs. 4, 5, and 6. Three adjacent sections of an area in which a polymorph is passing through endothelium.

In Fig. 4 two fragments of polymorph cytoplasm (1 and 2) lie in contact with endothelium, one (1) immediately above a complicated cell junction (J). No "cement" is apparent. A further fragment of polymorph (3) lies between endothelium and basement membrane (B).

In Fig. 5 continuity is seen between the polymorph fragments lying above and below the endothelium. The cell junction (J) appears intact.

In Fig. 6 the polymorph is seen penetrating the full thickness of endothelium down to basement membrane (B) but no cell junction is visible. Note the appearance of "flowing" of polymorph granules. All x 30,000.

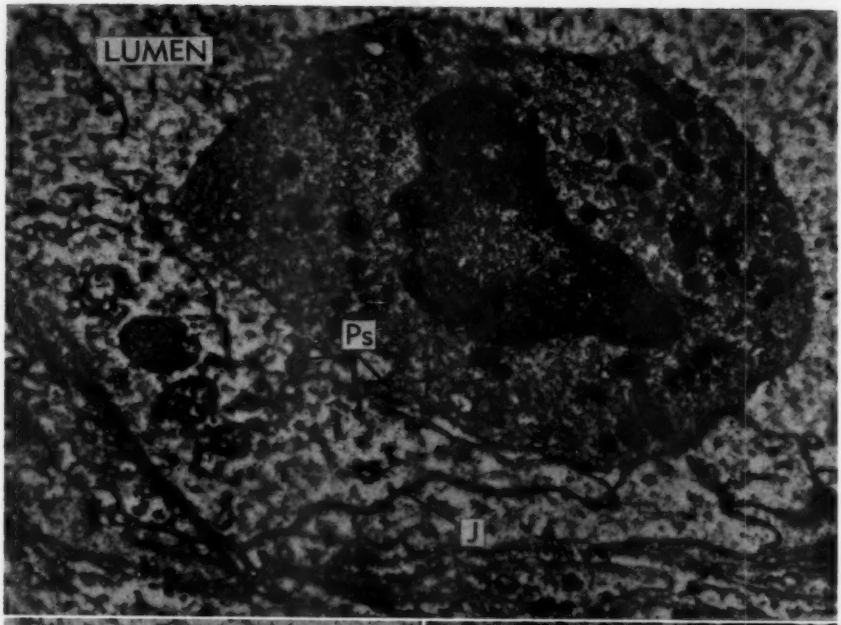
Figs. 7, 8, and 9. Three adjacent sections of an area in which a polymorph is passing through endothelium.

In Fig. 7 a polymorph separates two portions of endothelial cytoplasm in each of which a portion of nucleus (N) is visible. The penetration of the polymorph is limited by basement membrane (B) and peri-endothelial cells (PC). x 20,000.

In Fig. 8 large fragments of polymorph cytoplasm (1 and 2) lie below and one smaller fragment (3) above an endothelium in which a cell junction (J) cut tangentially is just visible. Nuclei are seen as in Fig. 7. x 24,000.

In Fig. 9 portion of the polymorph (1) is again present beneath an endothelium in which a cell junction (J) is clearly visible. The small fragment of cytoplasm (2) may be from a polymorph or from an adjacent endothelial cell. Nuclei present as before. x 24,000.

Fig. 1.



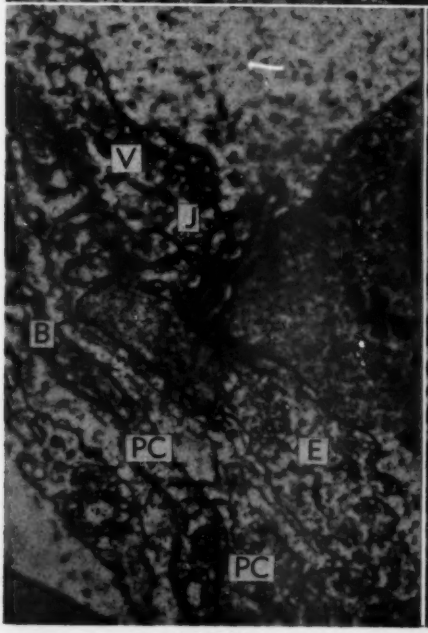
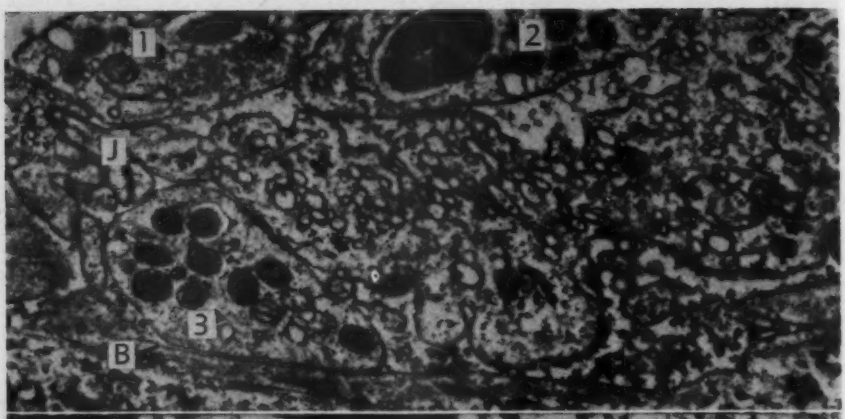


Fig. 2.

Fig. 3.



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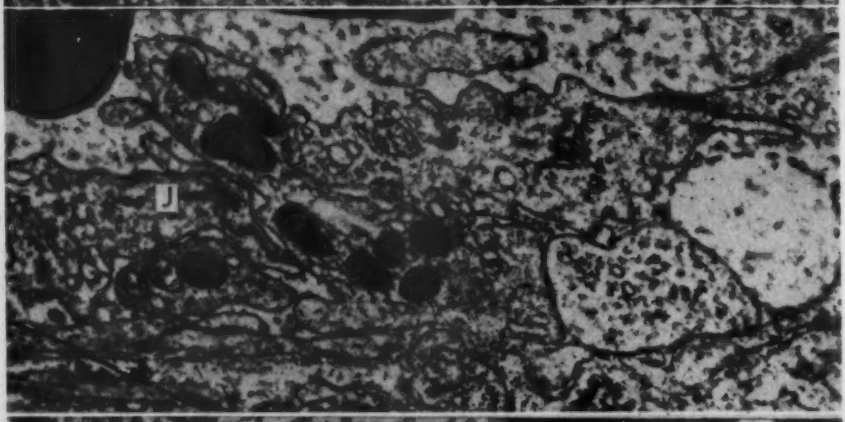

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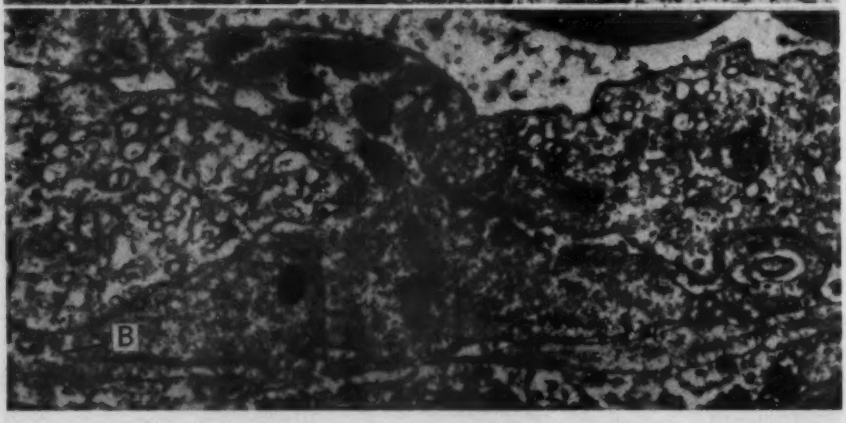

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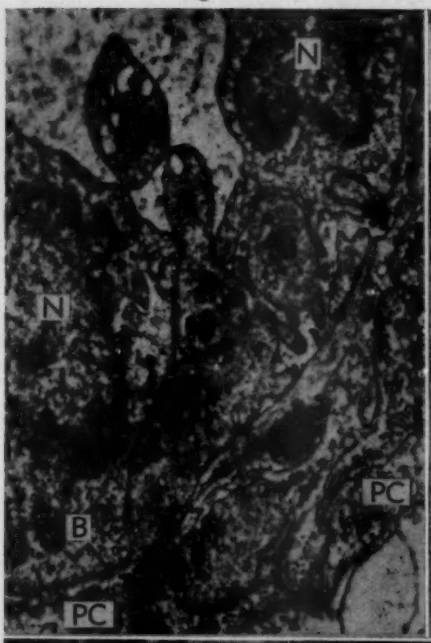
Fig. 7.



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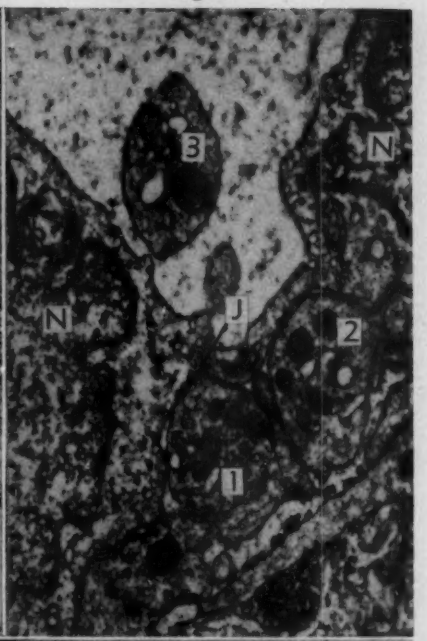


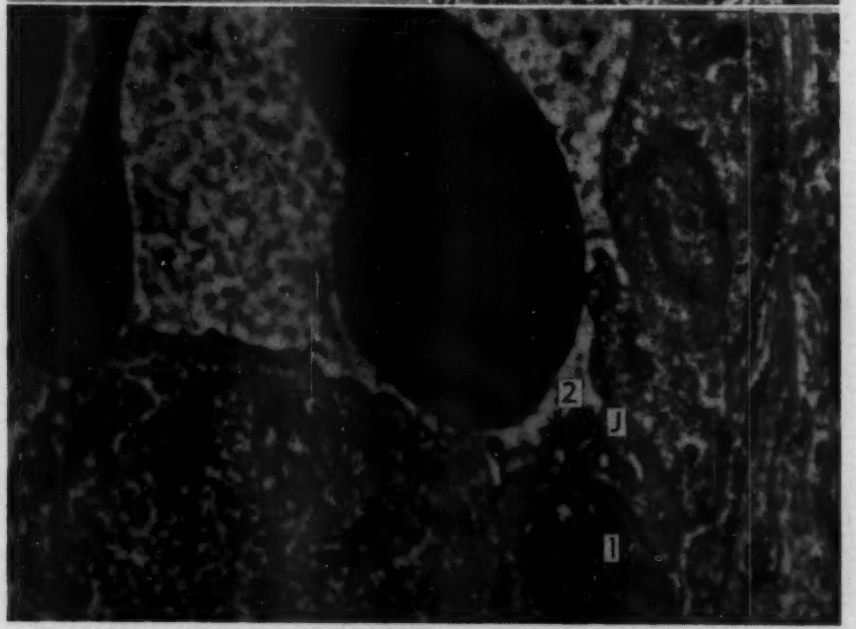


Fig. 9.

EXPLANATION OF PLATES.

Fig. 10. Portion of polymorph covered by endothelium except for a small gap (G). A poorly defined attachment belt is present in cell junction (J). x 16,000.

Fig. 11. Polymorph lying entirely between endothelium (E) and basement membrane (B). Two cell junctions (J) have no visible attachment belts. x 17,000.

Fig. 12. Polymorph lying between endothelium (E) and basement membrane (B) with a pseudopod (Ps) passing through basement membrane to abut directly against peri-endothelial cell (PC). Unidentified object (O) perhaps part of an active fibroblast is deep to basement membrane. x 16,000.

Fig. 13. Portion of polymorph (1) lying between basement membrane (B) and peri-endothelial cell (PC) with a smaller fragment of polymorph (2) deep to peri-endothelial cell. Dense object (R) within peri-endothelial cell is either portion of a red blood cell or a lipid inclusion. x 21,000.

Fig. 14. Polymorph, lying beneath basement membrane (B) and between two peri-endothelial cells (PC), has a pseudopod (Ps) passing into surrounding tissue. Note lines of caveolae (C) in both endothelial and peri-endothelial cells. x 16,000.

Fig. 15. Portion of a red blood cell (R) and two platelets (P) within a peri-endothelial cell. Note lines of caveolae (C) at lower border of the cell. x 20,000.

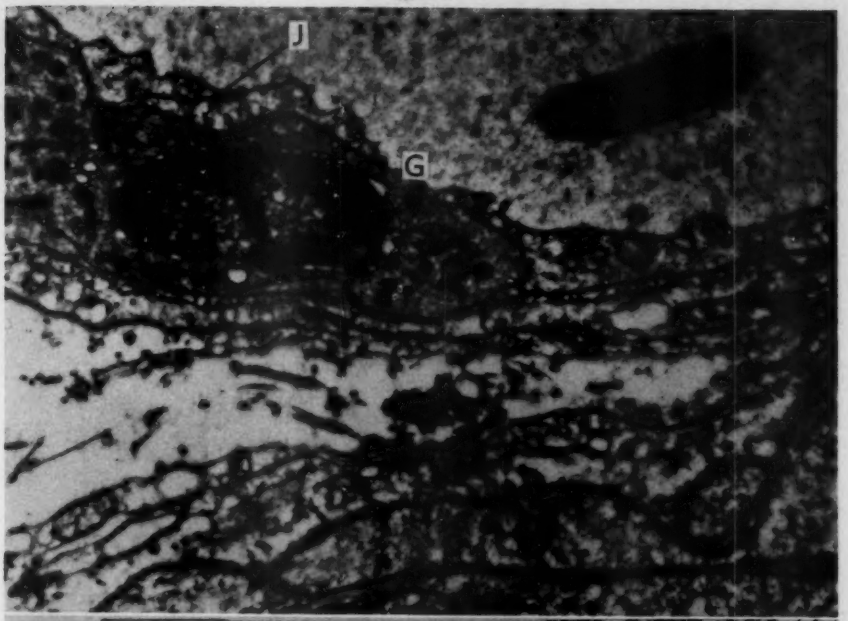
Fig. 16. Monocyte, with prominent mitochondria (M), lying in close contact with endothelium above a cell junction (J). No "cement" substance visible. Note depression (D) through almost full thickness of endothelium. x 18,000.

Fig. 17. Monocyte lying between endothelium and basement membrane (B). Fragment of polymorph (1) adherent to endothelium and part of another polymorph (2) lying outside vessel deep to peri-endothelial cell (PC). x 12,000.

Fig. 18. Monocyte lying beneath attenuated endothelial cytoplasm in which a cell junction (J) is seen. Passage through a gap in the basement membrane into peri-endothelial region is occurring. x 14,000.

Fig. 19. Portion of a monocyte lying deep to the peri-endothelial layer and almost completely surrounded by processes (1 and 2) of two peri-endothelial cells. x 19,000.

Fig. 10.



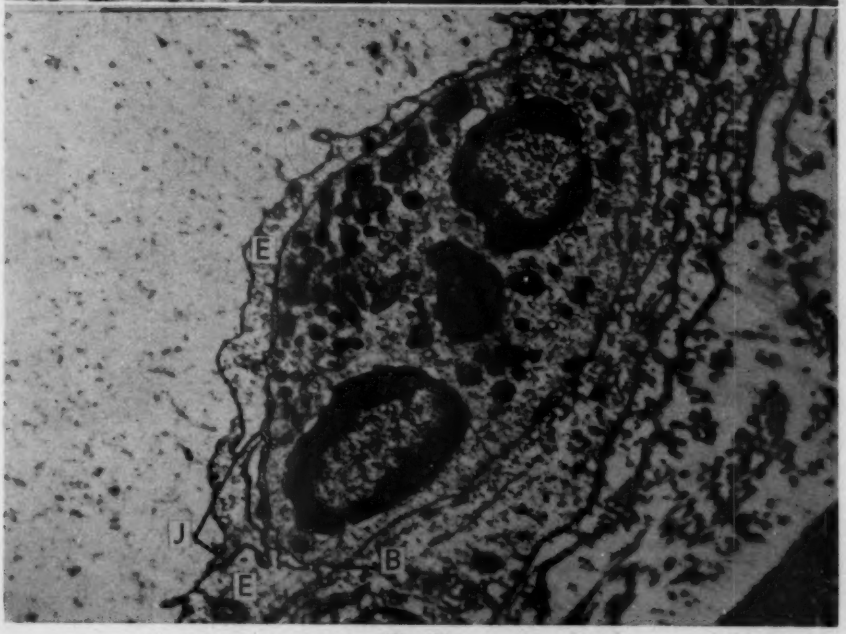
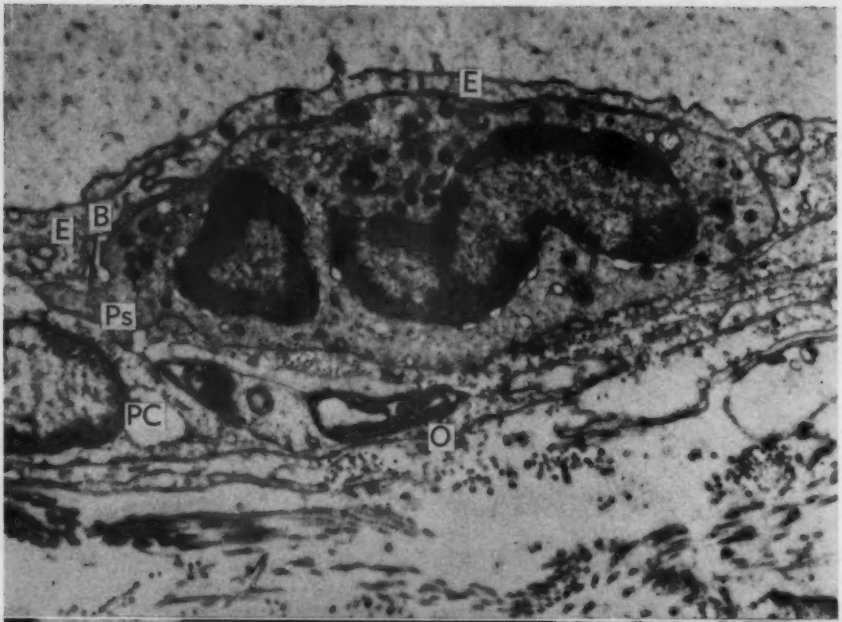


Fig. 11.

Fig. 12.



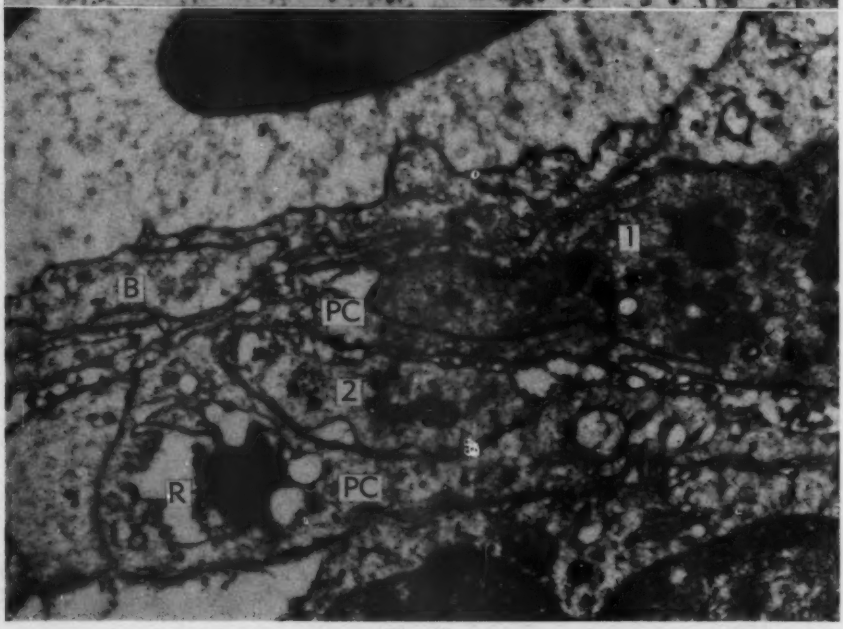


Fig. 13.

Fig. 14.

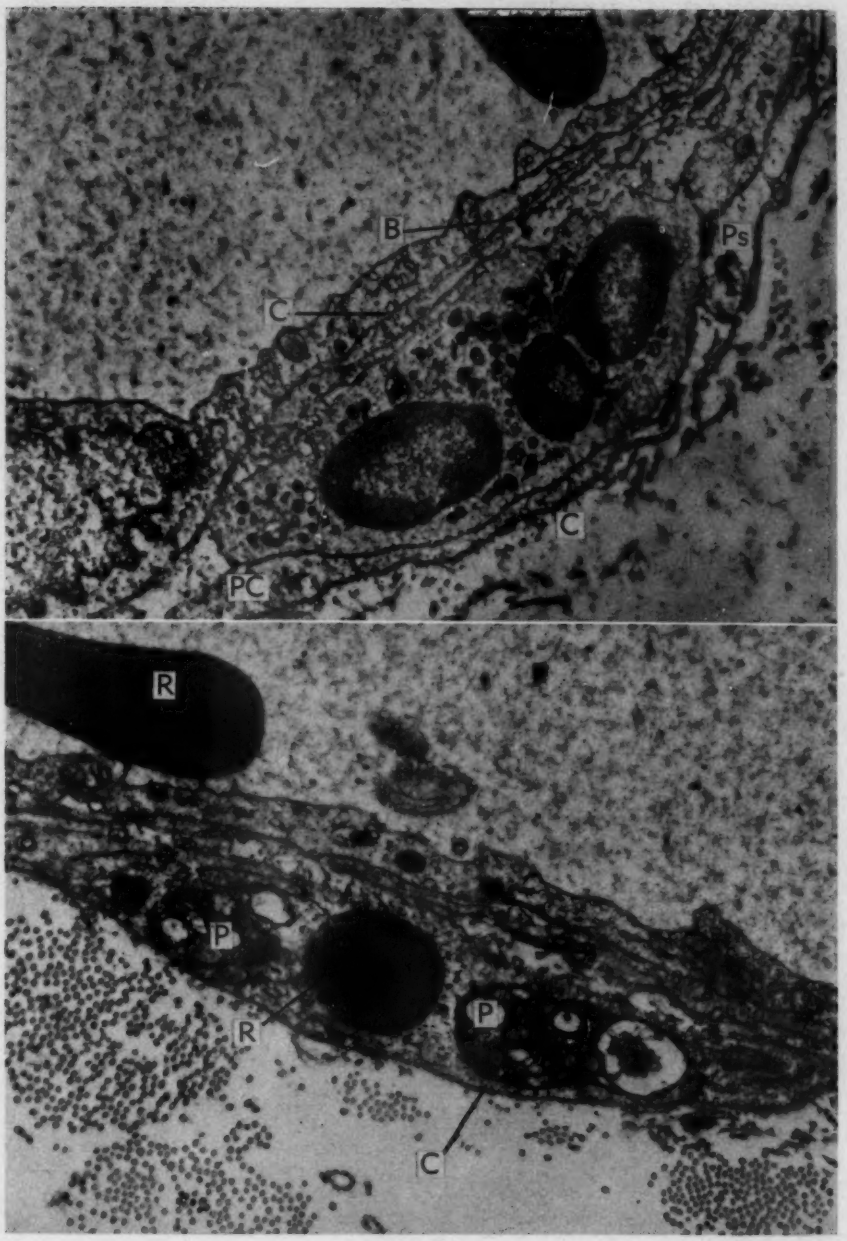
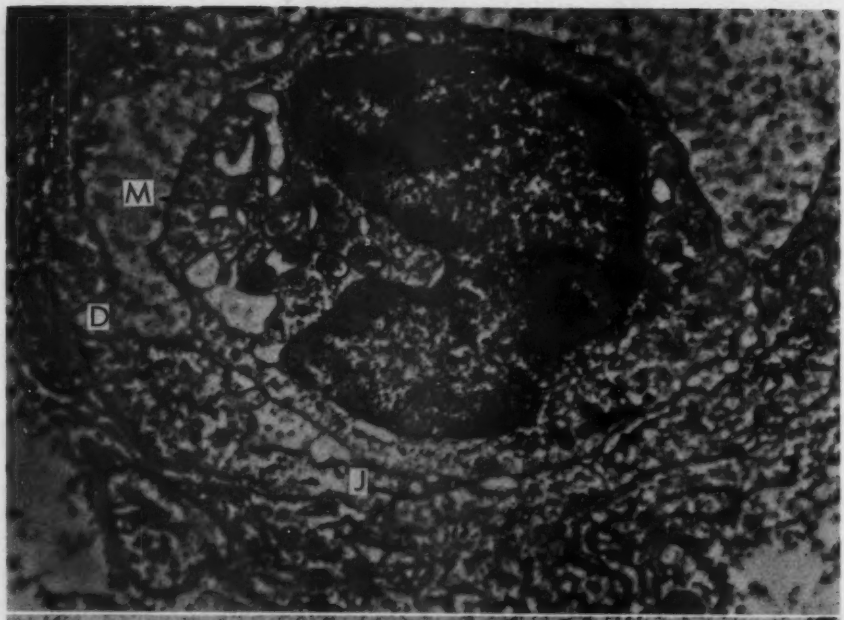


Fig. 15.

Fig. 16.



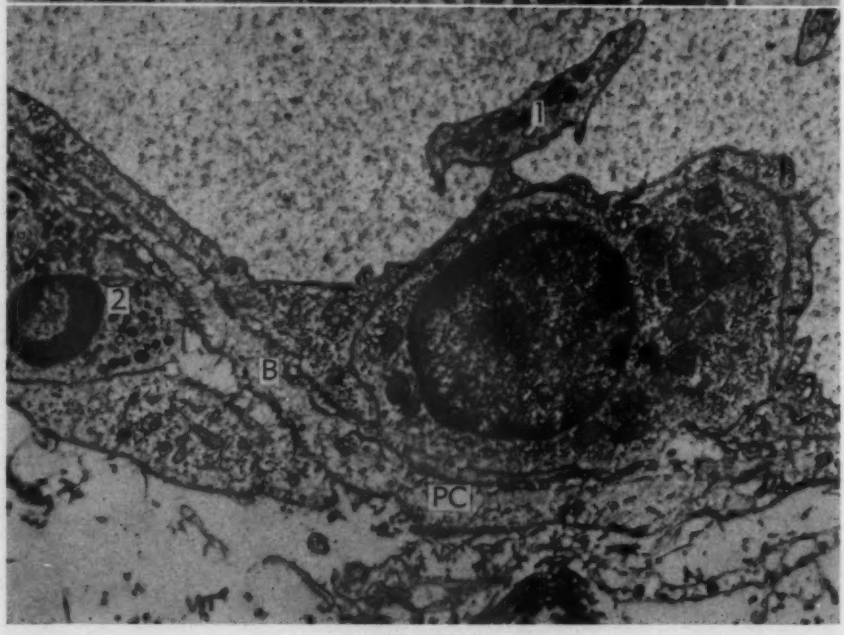


Fig. 17.

Fig. 18.

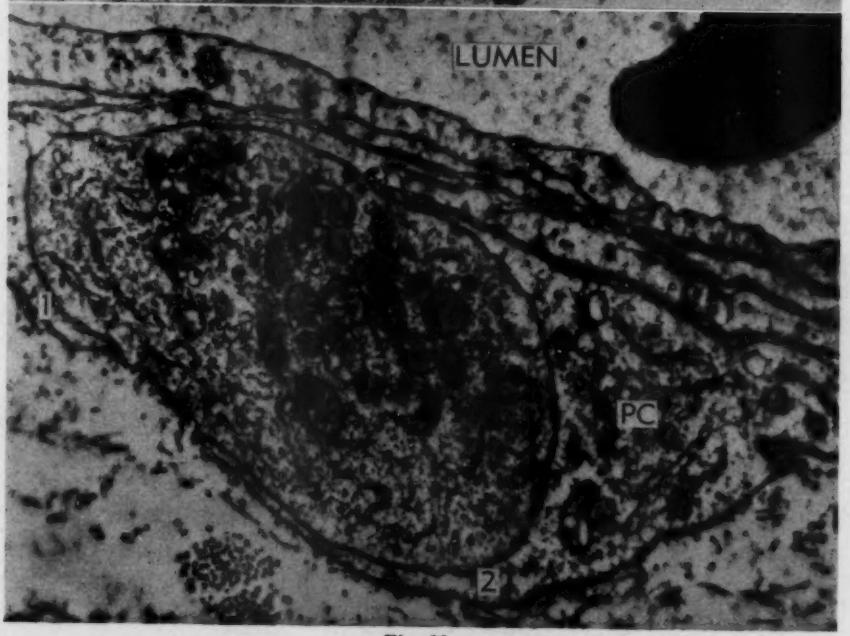


Fig. 19.

Red blood cells and platelets.

Portions of red blood cells and bodies packed with dense granules and small vesicles which were regarded as platelets are commonly visible within the lumen of capillaries and uncommonly within or between endothelial or peri-endothelial cells. In neither case were leucocytes seen migrating in relation to these intracellular structures, nor were the earlier stages of escape seen. Both erythrocytes and platelets may actually lie within other cells, but the same appearances could be produced by the plane of section passing through only portion of an erythrocyte (or platelet) which had invaginated the cytoplasm of an adjacent cell.

DISCUSSION.

The observations contained in this paper agree closely with the findings reported from Oxford of leucocytic emigration in rat mesentery (Marchesi and Florey, 1960; Marchesi, 1961) and in the tissue within ear chambers in the rabbit (Florey and Grant, 1961). The appearances in the stage of margination, the apparently rapid movement through the endothelium and the period of arrest between endothelium and subendothelial structures are the same in each case. With a few cells it appears to us proven that leucocytes as studied by serial section in our material migrated between adjacent cells – a process clearly demonstrated to occur in rat mesentery by Marchesi, but like him we are unable to prove that migration cannot also occur through the cytoplasm of individual endothelial cells. Only exhaustive serial sectioning could resolve this problem but all our findings are readily explicable by emigration occurring exclusively between cells, the several appearances seen being due to variation in the plane of sectioning.

The stimulus used in the present investigation produces massive leucocytic emigration with no concurrent increase in vascular permeability to water and protein; four hours after serum injection, when the specimens for electron microscopy were taken, vascular permeability has returned to normal (Hurley and Spector, 1961). No change was detected in the structure of the endothelial cells. Marchesi and Florey, using a mild mechanical stimulus, similarly found no change in the endothelium, whereas in vessels damaged by ultra-violet light Florey and Grant found definite and irregular changes in endothelium which they interpreted as due to a direct damaging effect of the stimulus. It seems clear that in both the rat and the rabbit leucocytic emigration can occur without concurrent visible change in endothelial structure from vessels situated in skin, mesentery or newly formed connective tissue. There is no present reason to doubt that emigration from small vessels wherever situated in these animals is essentially similar.

By contrast Williamson and Grisham (1961) describe a very different mechanism for the escape of leucocytes from the small blood vessels in the dog's pancreas. They conceive (see Text Fig. 1 in the above paper) the leuco-

cytes as being enmeshed in finger-like processes present on the inner surface of inflamed capillaries with subsequent escape of the cell by dissolution and reformation of basement membrane. Whilst appearances similar to their Text Fig. 1B and Fig. 6 have been seen in our material (Fig. 11), no free endothelial cell processes of the type they describe were observed. We have seen nothing to suggest that endothelial cells play the active rôle in emigration envisaged by these authors – appearances such as Figs. 10 and 17 are better explained by deformation of endothelial cells over a polymorph which has passed beneath the endothelium and been delayed by basement membrane or peri-endothelial structures. However, the presence of attenuated portions of endothelial cell cytoplasm extending into the lumen in contact with leucocytes passing through the endothelium, as shown in Fig. 3, strongly suggests that active movement of endothelial cell cytoplasm over the invading cell has occurred in contrast to the completely passive rôle of the endothelial cell envisaged by the Oxford workers.

Williamson and Grisham also describe large vesicles up to 1μ across in the endothelial cells of inflamed capillaries. Appearances of this type are not described in any of the Oxford papers, nor have we seen anything similar in our own material, both that described above and in as yet incomplete studies on capillaries in a state of greatly increased permeability. The explanation for the difference is not clear. It may be that in this regard the dog differs from the rat or the rabbit; alternatively the relatively gross stimulus used by Williamson and Grisham may have produced direct damage to capillary walls and the changes seen need not represent a necessary feature of the inflammatory response in the dog. At least some of the large vesicles in their Fig. 2 which contain small projections within them would appear to be the result of oblique sections of surface projections of endothelial cells and not intracellular structures. Further work is necessary to elucidate what part, if any, these vesicles play in the increased capillary permeability of the acute inflammatory response.

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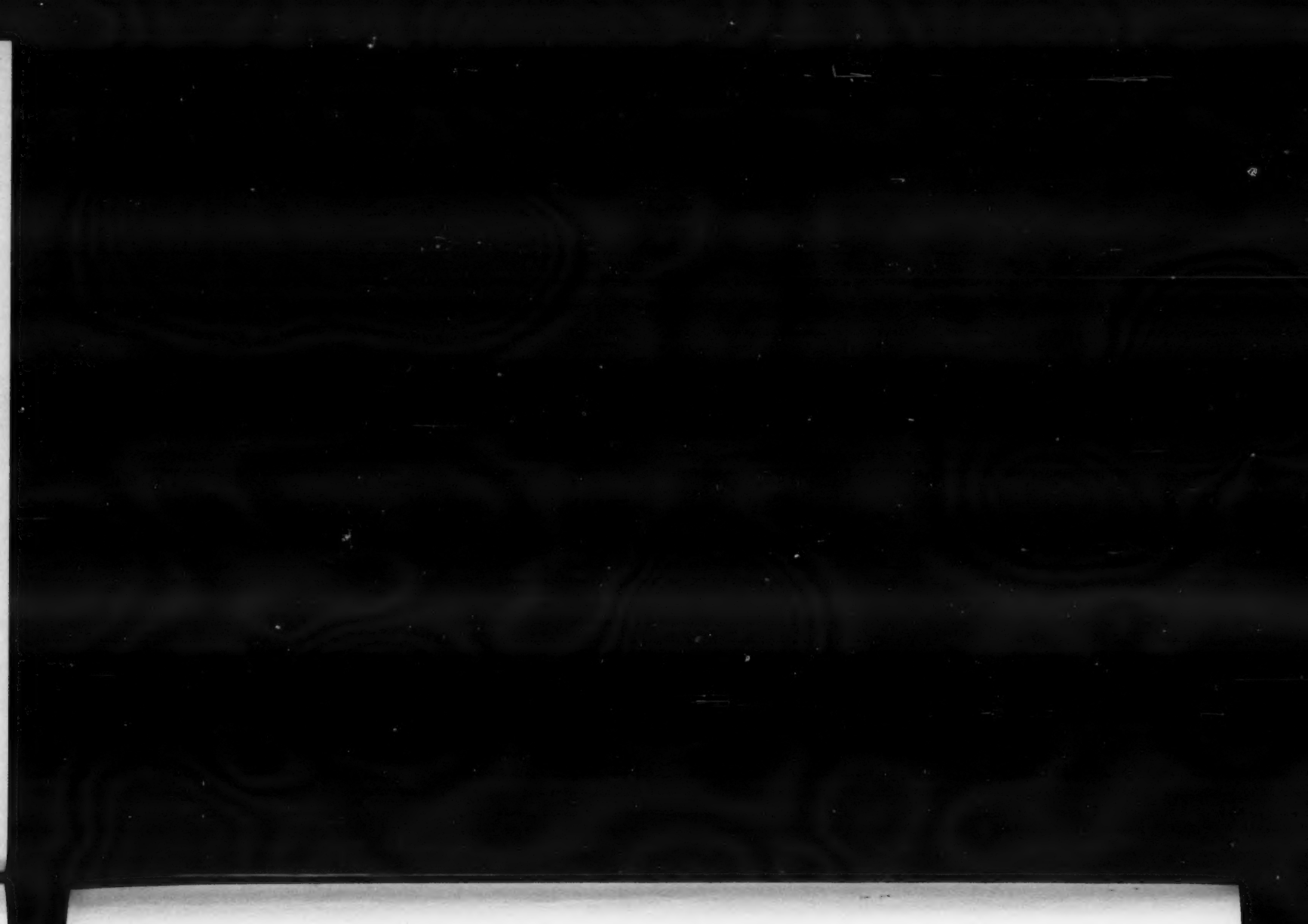
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